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13. ABSTRACT *(Maximum 200 Words)*

We have proposed to develop novel antibody fusion proteins in which antibodies specific for the breast cancer tumor associated antigen HER2/*neu* will be genetically fused to the cytokines IL-2, IL-12, and GM-CSF. It is expected that the anti-HER2/*neu* antibody cytokine fusion protein will localize the cytokine at the tumor and elicit an immune response. During our first year of funding we completed the construction and characterization of an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein. This novel molecule expressed in myeloma cells was correctly assembled and secreted and showed antibody and cytokine related activities. In addition, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu* expressing murine tumor CT26-HER2/*neu* and enhance the immune response against the targeted antigen, HER2/*neu,* suggesting its potential use in the treatment of HER2/*neu* expressing tumors. We also expanded our repertoire of human HER2/*neu* expressing murine cell lines by transducing the murine B cell lymphoma 38C13 with the cDNA encoding human HER2/*neu*. We studied the *in vivo* properties of this new tumor model (38C13-HER2/*neu*) and found that this model provides a useful tool for the evaluation of different therapeutic approaches designed to stimulate the tumor specific immune response.

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Sherri L. Morrison July 24, 2000
PI - Signature Date

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(5) INTRODUCTION

In the present grant we propose to explore the use of genetically engineered antibodies as therapeutic agents specifically attempting to augment and potentiate the host immune defense systems against breast cancer. We will use antibodies specific for HER2/*neu*, a molecule present on the surface of many breast cancers; its increased expression is associated with poor prognosis. To this antibody we will join the cytokines IL-2, IL-12, and GM-CSF. Expression of these cytokines by cancer cells has been shown to render them immunogenic. The anti-HER2/*neu* will be used to localize the cytokine at the tumor where it is expected to elicit an immune response. The resulting immune response would be expected to be specific not only for the targeting antigen, but also for other tumor associated antigens resulting in the destruction of both the tumor cells which express the targeting antigen as well as those that do not. Simultaneous targeting of more than one cytokine to the tumor would be expected to lead to synergism in immune activation and an even more potent immune response.

(6) BODY

During our first year of funding we completed the construction, expression and characterization (*in vitro* and *in vivo*) of an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein.

Anti-HER2/*neu* IgG3-(GM-CSF) expressed in myeloma cells was correctly assembled and secreted. Anti-HER2/*neu* IgG3-(GM-CSF) binds HER2/*neu* expressing cells with an affinity similar to that of the parental antibody. The GM-CSF in the fusion protein was able to support the growth of a GM-CSF dependent murine myeloid cell line, FDC-P1. The antibody fusion protein was comparable to the parental antibody in its ability to effect ADCC mediated tumor cell lysis and activate J774.2 macrophage cells so that they could lyse tumor cells in the absence of antibody. Pharmacokinetic studies showed that the half-life of anti-HER2/*neu* IgG3-(GM-CSF) depended on the injected dose with longer *in vivo* persistence observed at higher doses. Biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen. In addition, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu* expressing murine tumor CT26-HER2/*neu* and enhance the immune response against the targeted antigen HER2/*neu*. Anti-HER2/*neu* IgG3-(GM-CSF) is able to enhance both Th1 and Th2 mediated immune responses and treatment with this antibody fusion protein resulted in significant retardation in the growth of s.c. CT26-HER2/*neu* tumors. Our results indicate that anti-HER2/*neu* IgG3-(GM-CSF) fusion protein is useful in the treatment of HER2/*neu* expressing tumors.

We submitted a manuscript entitled "A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity" by Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet (1). It describes in detail the *in vitro* and *in vivo* properties of this novel molecule and is attached as an appendix.

Prior to the notification of funding of this proposal we completed the initial characterization of (IL-12)-anti-HER2/*neu* IgG3 (2). The publication describing these initial studies is attached as an appendix. Studies to define the mechanism of action of (IL-12)-anti-HER2/*neu* IgG3 will be done during the coming year.

To evaluate the immunological efficacy of the proposed antibody-cytokine fusion proteins, it is critical that tumors expressing the target antigen can grow in immunologically intact mice. To produce murine tumors expressing human HER2/*neu* on their surface, we transduced the murine cell lines CT26 (syngeneic to BALB/c), MC38 and EL4 (both syngeneic to C57BL/6) with a retroviral construct containing the cDNA encoding human HER2/*neu*. As explained in our original grant proposal, these three human-HER2/*neu* expressing cells were similar to their respective parental cell lines in histology and kinetics of tumor growth in subcutaneous space and more

important, they grow *in vivo* while maintaining the expression of human HER2/*neu*. The availability of different murine cell lines expressing human HER2/*neu* makes it possible to evaluate the effectiveness of HER2/*neu* targeted approaches in different cell lines and/or mouse strains. This is a very important issue because clear differences in the response to the same anti-cancer therapy are seen with different tumors and in different strains. For this reason, during our first year of funding we expanded our repertoire of human HER2/*neu* expressing murine cell lines by transducing the murine B cell lymphoma 38C13 (syngenic to C3H/HeN) with the cDNA encoding human HER2/*neu*. We studied the *in vivo* properties of this new tumor model (38C13-HER2/*neu*) and found that this new model differs in its behavior from our previously developed human HER2/*neu* expressing murine tumors (CT26-HER2/*neu*, MC38-CT26-HER2/*neu* and EL4-CT26-HER2/*neu*). We submitted a manuscript entitled "A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity" by Manuel L. Penichet, Jay S. Dela Cruz, Pia M. Challita-Eid, Joseph D. Rosenblatt and Sherie L. Morrison (3). The appended manuscript describes in detail the *in vitro* and *in vivo* properties of this cell line.

(7) KEY RESEARCH ACCOMPLISHMENTS

1) The construction and expression and characterization of anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein. No anti-human HER2/*neu* Ab (GM-CSF) fusion protein (IgG or scFv) has been reported.

2) The development and characterization of a new human HER2/*neu* expressing murine tumor model, the 38C13-HER2/*neu*.

(8) REPORTABLE OUTCOMES

Manuscripts:

Two manuscripts have been submitted:

1) "A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity" byJay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet.

2) "A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity" by Manuel L. Penichet, Jay S. Dela Cruz, Pia M. Challita-Eid, Joseph D. Rosenblatt and Sherie L. Morrison.

In both manuscripts we wrote the phrase: "This work was supported in part by Department of Defense Breast Cancer Research Program Grant BC980134."

Development of Cell Lines:

Two cell line have been developed and characterized:

TEAV: it is the murine myeloma cell line P3X63Ag8.653 expressing and secreting anti- HER2/*neu* IgG3-(GM-CSF).

TAUT: it is the murine B cell lymphoma 38C13 expressing on its surface human HER2/*neu*.

TEAV and TAUT are names assigned in our laboratory following our nomenclature system. However, in publications we usually use a descriptive name for cell lines. We have found that by so doing the manuscript is much easier to read.

(9) CONCLUSIONS:

1) Our results suggest that an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein containing human GM-CSF may be effective in patients with tumors overexpressing HER2/*neu*. The combination of an anti-HER2/*neu* antibody with GM-CSF yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down regulation of HER2/*neu* expression, ADCC and the stimulation of a strong anti-tumor immune response through the immunostimulatory activity of GM-CSF. In addition, the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein may be effective against tumor cells which express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* antibody therapy. Because of GM-CSF's ability to elicit an immune response to associated antigens, it is also possible that association of anti-HER2/*neu* IgG3-(GM-CSF) with soluble ECD^{HER2} shed by tumor cells will enhance the anti-tumor immune response.

More studies are now required to define the optimal dose and injection schedule for anti-HER2/*neu* IgG3-(GM-CSF), its mechanism of anti-tumor activity, the potential side effects, and to explore it is effectiveness against other human HER2/*neu* expressing murine tumor models . Similar studies are required for (IL-12)-anti-HER2/*neu* IgG3.

2) The new tumor model that we have developed (38C13-HER2/*neu*) provides a useful tool for the study of the mechanisms of protective immunity to B-cell lymphoma and for the evaluation of different therapeutic approaches based on the stimulation of the immune response.

(10) REFERENCES

1) Dela Cruz J.S., Trinh K.R., Morrison S.L., and Penichet M.L. A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity. 2000. Submitted.

2) Peng, L.S., Penichet, M.L., and Morrison, S.L. A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity. J. Immunol., 163:250-258, 1999.

3) Penichet M.L., Dela Cruz J.S., Challita-Eid P.M., Rosenblatt J.D., and Morrison S.L. A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity. 2000. Submitted.

(11) APPENDICES

The three manuscripts listed in section 10 (REFERENCES).

APPENDICES

1. Dela Cruz, J.S., Trinh, K.R., Morrison, S.L., and Penichet, M.L. A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity. 2000. Submitted.

2. Peng, L.S., Penichet, M.L., and Morrison, S.L. A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity. *J. Immunol.* 163:250-258. 1999.

3. Penichet, M.L., Dela Cruz, J.S., Challita-Eid, P.M., Rosenblatt, J.D., and Morrison, S.L. A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity. 2000 Submitted.

A recombinant anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein retains antigen specificity, cytokine function and demonstrates anti-tumor activity [1].

Jay S. Dela Cruz, K. Ryan Trinh, Sherie L. Morrison, and Manuel L. Penichet [2].

Departments of Microbiology and Molecular Genetics, and The Molecular Biology Institute. University of California, Los Angeles. 405 Hilgard Avenue, Los Angeles, CA 90095-1489.

Keywords: Antibodies; Cytokines; Immunotherapy; Cytotoxicity; In Vivo Animal Models

Running Title: **anti-human HER2/*neu* IgG3-(GM-CSF) fusion protein.**

Abstract

Anti-HER2/*neu* therapy of human HER2/*neu* expressing malignancies such as breast cancer has shown only partial success in clinical trials. To expand the clinical potential of this approach, we have genetically engineered an anti-HER2/*neu* IgG3 fusion protein containing granulocyte/macrophage colony-stimulating factor (GM-CSF). Anti-HER2/*neu* IgG3-(GM-CSF) expressed in myeloma cells was correctly assembled and secreted. It was able to target HER2/*neu* expressing cells and to support growth of a GM-CSF dependent murine myeloid cell line, FDC-P1. The antibody fusion protein was comparable to the parental antibody in its ability to effect ADCC mediated tumor cell lysis and activated J774.2 macrophage cells so that they could lyse tumor cells in the absence of antibody. Pharmacokinetic studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is stable in the blood. Interestingly the half-life of anti-HER2/*neu* IgG3-(GM-CSF) depended on the injected dose with longer *in vivo* persistance observed at higher doses. Biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen. In addition, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu* expressing murine tumor CT26-HER2/*neu* and enhance the immune response against the targeted antigen HER2/*neu*. Anti-HER2/*neu* IgG3-(GM-CSF) is able to enhance both Th1 and Th2 mediated immune responses and treatment with this antibody fusion protein resulted in significant retardation in the growth of s.c. CT26-HER2/*neu* tumors. Our results suggest that anti-HER2/*neu* IgG3-(GM-CSF) fusion protein will be useful in the treatment of HER2/*neu* expressing tumors.

Introduction

The *HER2/neu* proto-oncogene (also known as *c-erbB-2*) encodes a 185 kDa transmembrane glycoprotein receptor known as HER2/*neu* or $p185^{HER2}$ that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity (1-3). It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain (1-3). Overexpression of HER2/*neu* is found in 25-30 % of human breast cancer and this overexpression is an independent predictor of both relapse-free and overall survival in breast cancer patients (4-7). Overexpression of HER2/*neu* also has prognostic significance in patients with ovarian (5), gastric (8), endometrial (9), and salivary gland cancers (10). The increased occurrence of visceral metastasis and micrometastatic bone marrow disease in patients with HER2/*neu* overexpression has suggested a role for HER2/*neu* in metastasis (11, 12).

The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent TAA for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) previously known as rhuMAb HER2, directed at the extracellular domain of HER2/*neu* (ECD^{HER2}) (13) can lead to an objective response in some patients with tumors overexpressing the HER2/*neu* oncoprotein (14, 15). However, only a subset of patients shows an objective response [5 of the 43 (11.6%)] (14, 15). Although combination of trastuzumab with chemotherapy enhances its anti-tumor activity [9 of 37 patients with no complete response(24.3 %)]

(16), improved therapies are still needed for the treatment of HER2/*neu* expressing tumors.

GM-CSF is a cytokine associated with the growth and differentiation of hematopoietic cells. It is also a potent immunostimulator with pleiotropic effects, including the augmentation of antigen presentation in a variety of cells (17-22), increased expression of MHC class II on monocytes and adhesion molecules on granulocytes and monocytes (23-25), and amplification of T-cell proliferation (26). In animals, the injection of GM-CSF potentiates the protective effects of an anti-tumor vaccine by enhancing T-cell immunity (26) and vaccination with GM-CSF-transduced cells has been shown to be effective in the treatment of experimental tumors in murine models (27-30).

Studies suggest that for GM-CSF to be effective it must be concentrated in the vicinity of the tumor where it acts in a paracrine manner. A completed phase I clinical trial showed that vaccination of patients with metastatic melanoma with irradiated autologous melanoma cells engineered to secrete human GM-CSF stimulated potent antitumor immunity (31). Although the results suggest that this immunization strategy has potential application in the treatment of minimal residual disease, the *ex vivo* genetic modification and reintroduction of cells into patients is limited by its patient specific nature. Additionally, it is technically difficult, time consuming and expensive to expand primary autologous human tumor cells to the numbers required for vaccination (31-34). While *in vivo* gene delivery using viral vectors has been considered, the low transfer efficiency of retroviral vectors and the immunogenicity of adenoviral vectors has limited efficacy (34). Although systemic administration of GM-CSF is an alternative approach,

patients in clinical trials receiving high doses of GM-CSF have experienced severe toxic side effects (35) including a reported fatality (36) and no significant anti-tumor activity has been achieved. Thus the challenge of developing an effective approach for achieving high local concentrations of GM-CSF remains.

Antibody-(GM-CSF) fusion proteins that recognize tumor associated antigens provide one approach for achieving effective GM-CSF mediated immune stimulation at the site of the tumor. In the present report we characterize a novel antibody fusion protein, anti-HER2/*neu* IgG3-(GM-CSF) containing the variable region of the humanized anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) and the murine GM-CSF. The properties of anti-HER2/*neu* IgG3-(GM-CSF) suggest that it may provide an effective alternative for the therapy of HER2/*neu* expressing tumors.

Materials and Methods

Cell lines: CT26 is a murine colon adenocarcinoma that was induced in BALB/c mice by intrarectal injection of *N*-nitroso-*N*-methylurethane (37, 38). It was kindly provided by Dr. Young Chul Sung (Pohang University of Science and Technology, Korea). CT26-HER2/*neu* was developed in our laboratory by transduction of CT26 cells with the cDNA encoding human HER2/*neu* (39). We previously showed that this cell line is able to grow in immune competent mice while maintaining the expression of human HER2/*neu* on its surface (39).

J774.2, a macrophage cell line was obtained from Dr. Mathew Scharff (Albert Einstein College of Medicine, Bronx, NY). The P3X63Ag8.653 mouse non-producing

myeloma was purchased from the American Type Culture Collection (ATCC) (Rockville, MD). These four cell lines (CT26, CT26-HER2/*neu*, J774.2, and P3X63Ag8.653) were cultured in IMDM supplemented with 5% bovine calf serum, L-glutamine, penicillin, and streptomycin. The GM-CSF dependent murine myeloid cell line, FDC-P1, purchased from the American Type Culture Collection (ATCC) (Rockville, MD) was cultured in IMDM supplemented with 10% fetal bovine serum containing 25% WEHI-3 conditioned medium, L-glutamine, penicillin and streptomycin. All cells were incubated at 37°C in the presence of 5% CO_2.

Mice: Female BALB/c mice 6-8 weeks of age obtained from Taconic Farms, Inc. (Germantown, NY) were used. All experiments were performed according to National Institutes of Health (NIH) (Bethesda, MD) *Guide for the Care and Use of Laboratory Animals.* Animals were housed in a facility using autoclaved polycarbonate cages containing wood shaving bedding. The animals received food and water ad libitum. Artificial light was provided under a 12/12hrs light/dark cycle. The temperature of the facility was 20°C with 10-15 air exchanges per hr.

Vector construction, transfection and initial characterization of anti-human HER2/*neu* IgG3-C_H3-(GM-CSF): The DNA encoding the variable light (V_L) and heavy (V_H) chain domains of the humanized antibody hum4D5-8 (13) also known as trastuzumab (Herceptin; Genentech, San Francisco, CA) (15) or rhuMAb HER2 (14, 16) (generously provided by Paul Carter, Genentech, Inc., San Francisco, CA) had previously been cloned into mammalian expression

vectors for human kappa light chain and IgG3 heavy chain, respectively (40). The mature form of murine GM-CSF was amplified from the plasmid pCEP4/GM-CSF generously provided by Dr. Mi-Hua Tao (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan) by PCR using the sense primer 5'-CCCCTCGCGAGCGCACCCACCCGCTCACCC-3' and the antisense primer 5'-CCGAATTCGTTAACCTTTTTGGACTGGTTTTTTGCATTC-3'.

The PCR product was digested with NruI/EcoRI and cloned in the vector pAT3462 (previously developed in our laboratory) digested with SspI/EcoRI yielding the vector pAT1791 (Fig. 1). The plasmids pAT6611, pAH4874 (both previously developed in our laboratory), and pAT1791 were digested with EcoRV/NsiI, EcoRV/BamHI, and NsiI/BamHI respectively. The fragments containing the DNA encoding for anti-HER2/*neu* V_H and γ3 constant regions (from pAT6611), the expression vector backbone (from pAH4874), and GM-CSF (from pAT1791) were purified using Qiagen's (Chatsworth, CA) Gel Extraction Kit after electrophoresis in a 0.8% agarose gel. The three fragments were ligated yielding the anti-human HER2/*neu* IgG3-C_H3-(GM-CSF) heavy chain expression vector pAH1792. A cell line that produces high levels of anti-human HER2/*neu* kappa light chain, TAOL 5.2.3, was first obtained by transfecting P3X63Ag8.653 by electroporation with the mammalian expression vector for human anti-human HER2/*neu* kappa (Fig. 1), and selecting resistant mycophenolic acid stable transfectants. These were screened for L chain secretion by ELISA (enzyme-linked immunosorbent assay) (41). The heavy chain expression vector pAH1792 was used to electroporate the light chain producer TAOL 5.2.3 (Fig. 1). Stable transfectants were selected with 5 mM histidinol (Sigma Chemical, St. Louis, MO) and screened by ELISA for the secretion of heavy chain (41). Transfectants were biosynthetically labeled with ^{35}S-methionine (ICN, Irvine, CA) and the fusion protein

immunoprecipitated using rabbit anti-human IgG and a 10% suspension of staphylococcal protein A (IgGSorb, The Enzyme Center, Malden, MA) and analyzed by SDS-PAGE with or without reduction by β-ME. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma Chemical, St. Louis, MO). Protein concentrations were determined by bicinchoninic acid based protein assay (BCA Protein Assay, Pierce Chemical Co., Rockford, IL) and ELISA. Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The potential presence of aggregates in the purified protein was studied by FPLC (Superose 6, Amersham Pharmacia Biotech, Piscataway, NJ) in filtered and degassed PBS + 0.02% sodium azide.

Antigen binding: 10^6 CT26 or CT26-HER2/*neu* cells were incubated with 1 μg of anti-HER2/*neu* IgG3-(GM-CSF) in 0.1 ml of PBS plus 2% of bovine calf serum for 2 hrs at 4°C. Recombinant anti-HER2/*neu* IgG3 (40) and recombinant anti-DNS IgG3 antibodies were used as positive and negative isotype matched controls respectively. Cells were washed and incubated for 2 hrs at 4°C with 0.5 μg of biotinylated goat anti-human IgG (Pharmingen, San Diego, CA) in a volume of 0.1 ml of PBS plus 2% of bovine calf serum. Cells were washed and incubated for 30 min with 0.03 μg of PE-labeled streptavidin (Pharmingen, San Diego, CA) in a volume of 0.1 ml of PBS plus 2% of bovine calf serum. Analysis was performed by flow cytometry with a FACScan (Becton-Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Proliferation assay: The GM-CSF dependent murine myeloid cell line, FDC-P1 was used to study the bioactivity of anti-HER2/*neu* IgG3-(GM-CSF). rmGM-CSF from E. *coli* with $ED_{50} \leq 0.2$ ng/ml (Chemicon, Temecula, CA) reconstituted using deionized water following the manufacturer's recommendations and stored at - 20° C was used as reference standard. Serial 1:2 dilutions of equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF) were made in RPMI 1640 + 10% fetal bovine, over a range of 2 ng/ml to 16 pg/ml. Similarly, serial 1:2 dilutions of control anti-HER2/*neu* IgG3 were also included with a concentration equivalent to the antibody portion of anti-HER2/*neu* IgG3-(GM-CSF). 50 µl (5000 cells/well) of FDC-P1 myeloid cells in RPMI 1640 + 10% fetal bovine serum was mixed with 50 µl serial dilutions of rmGM-CSF, anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3, or medium in quadruplicate in a flat-bottom 96-well tissue culture plate (Costar, Corning, NY). After 48 hrs of culture at 37° C, 5% CO_2, proliferation was measured using the Cell Titer 96 aqueous non-radioactive colorimetric assay (Promega, Madison, WI) and plates were read at 490 nm.

Macrophage mediated cytotoxicity: Macrophage mediated cytotoxicity was performed according to the methods of Duerst *et al* (42) using the DNA fragmentation assay of Matzinger (43) with modifications. Briefly, the target cells CT26-HER2/*neu* were labeled with ^{3}H-thymidine (ICN, Irvine, CA) at 5 µCi/ml (sp: 6.7 Ci/mmol) in IMDM supplemented with 5% bovine calf serum for 24 hrs at 37°C. Labeled target cells were washed with medium and incubated with J774.2 macrophage effector cell in the presence of 5 µg/ml of anti-HER2/*neu* IgG3, the molar equivalent amount of anti-HER2/*neu* IgG3-

(GM-CSF) or no antibody for 24 hrs at 37° C. Alternatively J774.2 cells were incubated with 6.72 x 10^{-2} μg/ml of anti-HER2/*neu* IgG3-(GM-CSF)) (equivalent to 50U/mL of GM-CSF portion of anti-HER2/*neu* IgG3-(GM-CSF)), anti-HER2/*neu* IgG3 at a concentration equivalent to the antibody portion of anti-HER2/*neu* IgG3-(GM-CSF) (5.68 x 10^{-2} μg/ml) or with no additions in IMDM supplemented with 5% bovine calf serum for 24 hrs at 37°C. After incubation, the J774.2 cells were washed with medium and then transferred into a 96-well round bottom tissue culture plate (Costar, Corning, NY) containing 1 x 10^4 ^{3}H-thymidine labeled CT26-HER2/*neu* per well (effector:target ratio of 10). All incubations were carried out for 24 hrs in a final volume of 200 μl per well using IMDM supplemented with 5% bovine calf serum and 50 μM cold thymidine. The presence of 50 μM cold thymidine blocks the incorporation of released ^{3}H-thymidine by the J774.2 effector cells (42). The cells were harvested and passed through a glass-fiber filter (Wallac Oy, Turku, Finland) using a Micro Cell Harvester (Skatron, Norway). Labeled DNA from intact target cells was captured by the filters. The radioactivity was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The percent cytotoxicity mediated by J774.2 macrophage cells was calculated by the formula: ((cpm control - cpm test)/ cpm control) x 100; where cpm control represents ^{3}H measured in the wells containing target cells and anti-HER2/*neu* IgG3, anti-HER2/*neu* IgG3-(GM-CSF) or medium but lacking J774.2 macrophage cells. cpm test represents wells containing target cells in the presence of either effector cells pre-incubated with anti-HER2/*neu* IgG3, or anti-HER2/*neu* IgG3-(GM-CSF) or neither and

antibodies (anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF)). All assays were done in quadruplicate.

Half-life: Anti-HER2/*neu* IgG3-(GM-CSF) was iodinated to approximately 2 μCi/μg with ^{125}I using Iodobeads (Pierce, Rockford, IL) according to manufacturer's protocol. Mice were injected i.v. via the lateral tail vein with 1 μCi of ^{125}I-labeled proteins alone or mixed with 20 μg of cold anti-HER2/*neu* IgG3-(GM-CSF). At various intervals after injection of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) residual radioactivity was measured using a mouse whole body counter (Wm. B. Johnson & Assoc. Inc., Montville, NJ). Blood samples were obtained from the tail vein of mice 2, 4 and 12 hrs after injection. Serum was separated from clotted blood and stored at -20°C until assayed by SDS-PAGE to confirm the integrity of the protein.

Biodistribution: Groups of 4 mice were sacrificed 4 hrs or 16 hrs after the i.v. injection of 1 μCi (0.5 μg) of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Various organs and blood were collected, weighed and radioactivity measured using a gamma counter (Gamma 5500, Beckman Coulter, Inc., Fullerton, CA). Data are presented as percent of injected dose per gram of tissue (%ID/g tissue). Values were corrected for the radioactivity in blood in each tissue using the values of blood volume corresponding to each organ (44).

Tumor targeting: Anti-HER2/*neu* IgG3-(GM-CSF) was iodinated as described above. 10^6 CT26 and CT26-HER2/*neu* cells in 0.15 ml of Hank's Balanced Salt Solution (HBSS)

(GibcoBRL, Grand Island, NY) were injected separately into the left and right flanks of 3 mice. Seven days after tumor injection when tumors were about 1.0 cm in diameter, the 3 mice were injected i.v. via the lateral tail vein with 6 μCi of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Mice were euthanized 12 hrs after injection of anti-HER2/*neu* IgG3-(GM-CSF). Tumors and blood were removed, weighed and radioactivity measured using a gamma counter. Data are presented as percent of injected dose per gram of tumor (%ID/g tumor).

Immunotherapy: 1 x 10^6 CT26-HER2/*neu* cells in 0.15 ml HBSS were injected s.c. into the right flank of syngeneic BALB/c mice. Beginning the next day mice randomized into groups of 8 received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized. Blood samples were collected, serum was separated from clotted blood and stored at -20°C until assayed by ELISA.

Determination of murine anti-human HER2/*neu* and anti-human IgG3 antibodies: Sera from each treatment group were analyzed by ELISA for the presence of antibodies to human IgG3 and human HER2/*neu* using 96-well microtiter plates coated with 50 μl of anti-human HER2/*neu* IgG3 or human ECD^{HER2} (at a concentration of 1 μg /ml) respectively. The plates were blocked with 3% BSA in PBS and dilutions of serum in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. After

washing with PBS, alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) was added and the plates were incubated for 1 hr at 37 °C. After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma Chemical, St. Louis, MO) was added to the wells for 1 hr and plates were read at 410 nm. Sera from mice of the same age bearing tumors of the parental cell line CT26 was used as a negative control for determining anti-HER2/*neu* titers. Sera from naïve mice of the same age were used as a negative control for determining anti-human IgG3 titers. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

Determination of isotype profile of murine anti-human HER2/*neu* and anti-human IgG3 antibodies: The isotype of the murine anti-human IgG3 and anti-human HER2/*neu* was determined by ELISA using 96-well microtiter plates prepared as described above. Pooled sera from each treatment group diluted 1:50 in 1% BSA in PBS was added at 50 µl per well in duplicate into the 96-well plates and allowed to stand overnight at 4°C. After washing the plates with PBS, rat antibodies specific for murine IgG2a, IgG2b, IgG3, IgG1 or kappa (Pharmingen, San Diego, CA) diluted in 1% BSA in PBS were added to each well and incubated 2 hrs at room temperature. After washing with PBS, alkaline phosphatase (AP)-labeled goat anti-rat IgG (Pharmingen, San Diego, CA) was added and the plates were processed as described above.

Statistical analysis: Statistical analysis of the titration ELISA was made using the Mann-Whitney Rank test and the statistical analysis of the DNA fragmentation assay and the anti-tumor experiments was done using a two-tailed Student's *t*-test. For all cases results were regarded significant if p values were ≤ 0.05.

Results

Construction, expression, and initial *in vitro* characterization of anti-HER2/*neu* IgG3-C_H3-(GM-CSF): The strategy for the construction and expression of anti-HER2/*neu* IgG3-C_H3-(GM-CSF) is illustrated in Fig. 1. Clones expressing anti-HER2/*neu* IgG3-C_H3-(GM-CSF) were identified by ELISA and biosynthetically labeled by growth in the presence of ^{35}S-methionine. Labeled secreted protein was immunoprecipitated using rabbit anti-human IgG and analyzed by SDS-PAGE under reducing and non-reducing conditions. The anti-HER2/*neu* IgG3-C_H3-(GM-CSF) was correctly assembled and secreted and exhibits the expected molecular weight (data not shown). These results were confirmed by SDS-PAGE of purified proteins. In the absence of reducing agents anti-HER2/*neu* IgG3 migrates with an apparent molecular weight of 170 kDa while anti-HER2/*neu* IgG3-(GM-CSF) is about 200 kDa, the size expected for a complete IgG3 with 2 molecules of GM-CSF attached (Fig. 2, panel A). Following treatment with the reducing agent, light chains migrating with an apparent m.w. of approximately 25 kDa are seen for both proteins. However, the anti-HER2/*neu* IgG3 has a heavy chain of approximately 60 kDa, while anti-HER2/*neu* IgG3-(GM-CSF) has a heavy chain of approximately 75 kDa (Fig 2, panel B) as expected. Thus, proteins of the expected m.w. are produced and fusion

of murine GM-CSF to the carboxy terminus of the heavy chain of anti-HER2/*neu* IgG3 does not appear to alter the assembly and secretion of the H_2L_2 form of the antibody fusion protein. Analysis of anti-HER2/*neu* IgG3 and anti-HER2/*neu* IgG3-(GM-CSF) by FPLC under non-denaturing conditions showed that both proteins eluted as a single peak of the expected m.w. with no evidence of aggregation (data not shown).

Antigen binding at the cell surface: The ability of anti-HER2/*neu* IgG3-(GM-CSF) to bind to the HER2/*neu* target antigen was examined using flow cytometry. Both anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3 specifically bound to the human HER2/*neu* expressed on the surface of the murine cell line CT26-HER2/*neu* (Fig 3, panels B and C). Importantly, the same fluorescence intensity was seen suggesting that they have the same affinity for HER2/*neu.* No non-specific binding to CT26 that does not express HER2/*neu* was observed (Fig. 3, panels E and F).

Proliferation assay: Anti-HER2/*neu* IgG3-(GM-CSF) was able to specifically stimulate the proliferation of the GM-CSF dependent cell line FDC-P1. The proliferative response to equimolar GM-CSF concentrations of either rmGM-CSF or the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein was similar (Fig. 4). No proliferation was detected when cells were incubated with the same amount of anti-HER2/*neu* IgG3 (data not shown). The GM-CSF activity of anti-HER2/*neu* IgG3-(GM-CSF) present in culture supernatants was similar to that of purified protein indicating that the low pH used for elution from protein A does not reduce GM-CSF activity (data not shown).

Macrophage mediated cytotoxicity: Two assays were used to examine the ability of anti-HER2/*neu* IgG3-(GM-CSF) to augment macrophage mediated killing of tumor cells. Tumor cells and the macrophage cell line J774.2. were incubated 24 hrs in the presence of 5 μg/ml of anti-HER2/*neu* IgG3 or the molar equivalent of anti-HER2/*neu* IgG3-(GM-CSF). Equivalent tumor cell lysis was seen with both proteins indicating that the Fc region of the fusion protein can be bound by the macrophage receptors to elicit ADCC (Fig. 5, panel A). The tumor cell lysis observed with the incubation of anti-HER2/*neu* IgG3 or anti-HER2/*neu* IgG3-(GM-CSF) was statistically significant when compared with the results obtained with the incubation of the effector and target cells in absence of the antibody ($p < 0.05$). In the second assay effector cells were incubated with 6.72×10^{-2} μg/ml anti-HER2/*neu* IgG3-(GM-CSF), anti-HER2/*neu* IgG3 or medium alone, washed to remove unbound antibody or fusion protein, and then incubated with labeled target cells for 24 hrs. Anti-HER2/*neu* IgG3-(GM-CSF) treated J774.2 cells were significantly ($p < 0.0002$) more effective in lysing tumor cells than were effector cells activated in presence of anti-HER2/*neu* IgG3 (Fig. 5, panel B) which were similar to effector cells incubated in the absence of antibodies. Therefore the GM-CSF in the fusion protein retains the ability to mediate macrophage activation.

Half-life: The half-life of ^{125}I-labeled anti-HER2/*neu* IgG3 and anti-HER2/*neu* IgG3-(GM-CSF) was examined in BALB/c mice. Mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) of ^{125}I-labeled protein and the residual radioactivity measured using a

mouse whole body counter. Anti-HER2/*neu* IgG3 exhibited a half-life of 110 hrs, similar to what had previously been observed with chimeric IgG3 (45) (Fig. 6). Anti-HER2/*neu* IgG3-(GM-CSF) cleared more rapidly with a half-life of approximately 2 hrs indicating that fusion of the murine GM-CSF to the human anti-HER2/*neu* IgG3 significantly decreases the half-life. However, since we plan to treat the mice with a much higher dose (20 μg) of anti-HER2/*neu* IgG3-(GM-CSF) we also studied the half-life when this amount of protein was injected by mixing 20 μg of cold anti-HER2/*neu* IgG3-(GM-CSF) with 1 μCi (0.5 μg) of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) prior to injection. Increasing the quantity of injected anti-HER2/*neu* IgG3-(GM-CSF) injected increased the half-life five to six fold (10-12 hrs) (Fig. 6). Although results shown in Fig. 6 represent the mean of only two mice per group similar results were obtained when this experiment was repeated (data not shown).

Sera obtained from each mouse 2, 4 and 12 hrs after injection were fractionated without reduction on SDS-PAGE and examined by autoradiography. The radioactivity was present at the position expected for intact protein with the intensity of the band correlating with the residual radioactivity determined by whole body counting.

Biodistribution: Groups of 4 mice injected i.v. via the lateral tail vein with 1 μCi of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) were euthanized 4 hrs (time equivalent to 2 half-lives of the injected protein) or 16 hrs after injection. Various organs and blood were collected, weighed and radioactivity measured using a gamma counter. 4 hrs after its injection anti-HER2/*neu* IgG3-(GM-CSF) shows targeting to the spleen, followed by the

kidneys, liver and lungs (Fig. 7A). By 16 hrs after the injection, most of anti-HER2/*neu* IgG3-(GM-CSF) had cleared with some radioactivity remaining in the spleen, kidneys and blood. Splenic uptake may reflect the large number of GM-CSF receptor bearing cells in this organ. The presence of radioactivity in the kidneys and liver, sites of degradation and elimination, is consistent with the rapid elimination of anti-HER2/*neu* IgG3-(GM-CSF).

Tumor targeting: To examine the tumor targeting capability of anti-HER2/*neu* IgG3-(GM-CSF), BALB/c mice were injected with 10^6 CT26 and CT26-HER2/*neu* tumor cells in the left and right flanks, respectively. Seven days after tumor injection when tumors were about 1.0 cm in diameter, groups of 3 mice were injected i.v. via the lateral tail vein with 6 μCi of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). The mice were euthanized 12 hrs later, the tumors and blood were removed and weighed and the ^{125}I-labeled protein present was measured by a gamma counter. In all mice, enhanced localization of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) was seen in the CT26-HER2/*neu* tumor compared to CT26 that did not express HER2/*neu* (Fig. 8). These data indicate that anti-HER2/*neu* IgG3-(GM-CSF) is able to specifically target HER2/*neu* expressing cells.

Anti-tumor activity: To investigate *in vivo* anti-tumor activity, 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day mice were randomized and groups of 8 received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Injection of anti-HER2/*neu* IgG3-(GM-CSF) results in a

significant retardation in the tumor growth in most of the mice as compared with the respective controls of PBS or anti-HER2/*neu* IgG3 (Fig. 9, Experiment 1). When the experiment was repeated similar results were obtained (Fig. 9, Experiment 2). When the data of Experiments 1 and 2 were pooled, treatment with anti-HER2/*neu* IgG3-(GM-CSF) was found to result in highly significant anti-tumor activity ($p \leq 0.02$) for all the observed points (Table I). There was no statistically significant difference in tumor volume between the groups injected with PBS and anti-HER2/*neu* IgG3

Murine antibody response to HER2/*neu* and human IgG3: Sera from all mice in Experiment 2 were analyzed for the presence of antibodies recognizing the TAA HER2/*neu* and the human IgG3 antibody used for treatment. Mice treated with anti-HER2/*neu* IgG3-(GM-CSF) exhibited a significantly increased antibody response to both HER2/*neu* ($p < 0.04$) and human IgG3 ($p < 0.001$) compared to mice treated with either PBS or anti-HER2/*neu* IgG3 (Table II).

Isotype of murine antibody response: To further characterize the antibody response, the relative levels of the different isotypes present in the serum of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3 treated mice were determined (Fig. 10). Mice treated with anti-HER2/*neu* IgG3-(GM-CSF) showed significantly higher levels of all isotypes (with the exception of IgG3) recognizing human IgG3 when compared to anti-HER2/*neu* IgG3 treated mice (Fig. 10, Panel A). The increase in antibodies of the γ2a and γ1 isotypes suggests activation of both Th1 and Th2 mediated responses against this antigen

respectively. When antibodies directed against HER2/*neu* were examined (Fig. 10, panel B), animals treated with anti-HER2/*neu* IgG3-(GM-CSF) showed an increase in γ2b and γ1 but not γ3 and γ2a compared to animals treated with anti-HER2/*neu* IgG3. Thus the increased antibody response to HER2/*neu* was predominately of the isotypes characteristic of the Th2 response.

Discussion

In an attempt to improve the clinical efficacy of anti-HER2/*neu* based therapies we have developed an alternative approach in which a human IgG3 containing the variable regions of trastuzumab (Herceptin, Genentech, San Francisco, CA) has been genetically fused to potent immunostimulatory molecules such as the cytokine IL-12 (46) and the costimulatory molecule B7.1 (40). In the present study we expand this family of anti-HER2/*neu* antibody fusion proteins to include a fusion with the important cytokine GM-CSF.

A number of factors were considered in the design of our anti-HER2/*neu* IgG3-(GM-CSF) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility thereby facilitating simultaneous antigen and receptor binding (47, 48). IgG3 is also effective in complement activation (49), and binds FcγRs (50). GM-CSF was used because of its potent immunostimulating properties and ability to serve as a strong potentiator of tumor vaccines (26-30). Although our long-term goal is the production of antibody fusion proteins for therapeutic use in humans, human GM-CSF is not active in mice (35). Therefore we used murine GM-CSF in our fusion

protein so that we could perform *in vivo* studies using immune competent mice. We found that anti-HER2/*neu* IgG3-(GM-CSF) retains the ability to bind HER2/*neu* while the murine GM-CSF attached to the carboxy terminus of each heavy chain remains active.

In addition to the antibody-induced down regulation of HER2/*neu* expression ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (15). Indeed recent studies have indicated that ADCC is an important effector mechanism for antibody mediated tumor rejection (51). Fusion of GM-CSF to the carboxy terminus of C_H3 did not interfere with the antibody's ability to mediate ADCC (Fig 5, panel A) or activate macrophages so that they exhibited antibody independent cytotoxicity (Fig. 5, panel B). A recombinant fusion protein with a human-mouse chimeric IgG1 specific for B-cell malignancies fused to human GM-CSF (chCLL-1/GM-CSF) showed enhanced ADCC activity using human mononuclear cells compared with antibody (chCLL-1) alone (52). It is therefore possible that an anti-HER2/*neu* IgG3-(GM-CSF) containing human GM-CSF will exhibit superior anti-tumor activity. In addition directing GM-CSF to the tumor microenvironment using anti-HER2/*neu* IgG3-(GM-CSF) may lead to enhanced macrophage activation at the site of the tumor; in murine models, activated macrophages given locally and intravenously inhibit tumor growth and decrease metastatic development (53).

Systemic clearance of anti-HER2/*neu* IgG3-(GM-CSF) is rapid compared to anti-HER2/*neu* IgG3. This is consistent with what has been observed with other antibody cytokine fusion proteins (54) demonstrating a dominant role for the attached cytokine in determining the pharmacokinetics of the fusion proteins. We believe that the rapid

clearance of the antibody fusion protein is through the GM-CSF receptors on normal cells (35) such as splenic T-cells, B-cells and macrophages (55). In fact, our biodistribution studies showed that anti-HER2/*neu* IgG3-(GM-CSF) is mainly localized in the spleen consistent with earlier reports for the site targeted by murine GM-CSF (56). Interestingly we found a dose-dependent rate of clearance with rapid clearance ($t_{1/2}$=2 hrs) seen when 0.5 μg was injected and slower clearance ($t_{1/2}$= 10-12 hrs) when 20 μg was injected. It may be that the higher doses saturated the available GM-CSF receptors. It has yet to be determined in patients if the kinetics of clearance of anti-HER2/*neu* IgG3-(GM-CSF) will depend on the dose administered although in a clinical study using non-glycosylated human GM-CSF injected i.v. no clear relationship between dose and half-life was observed (57). Despite its rapid clearance, anti-HER2/*neu* IgG3-(GM-CSF) retains the capacity to effectively target the tumor. In fact, the rapid clearance may be beneficial in clinical applications in which potentially injurious cytokine exposure to normal tissues should be minimized.

A half-life of approximately 30 hrs has been reported for the chCLL-1/GM-CSF fusion protein injected i.p (52). The difference in clearance rates between anti-HER2/*neu* IgG3-(GM-CSF) and chCLL-1/GM-CSF may be explained by the use of different doses, by the route of injection (i.v. and i.p. respectively) and/or by the nature of the GM-CSF which were murine and human respectively. Murine GM-CSF has considerably higher affinity for the murine GM-CSF receptor than does human GM-CSF (58) which may lead to more rapid clearance. A GM-CSF fusion protein specific for the murine transferrin

receptor had a half-life of approximately 1.8 hrs (59). In this case it is likely that the antibody fusion proteins were rapidly cleared by the ubiquitous transferrin receptor (60).

We have found that treatment with anti-HER2/*neu* IgG3-(GM-CSF) causes a significant retardation in the growth of s.c. CT26-HER2/*neu* tumors under conditions in which anti-HER2/*neu* failed to confer protection. Our data are consistent with earlier experiments in which ch17217-(murine GM-CSF) specific for the murine transferrin receptor suppressed the development of pulmonary metastasis in 5/8 immune competent mice injected with CT26. However it should be noted that the control of antibody alone (ch17217) was not included in these earlier studies making it impossible to distinguish the role of the antibody from that of GM-CSF (59).

Several factors could explain our failure to obtain complete tumor remission. The dose, route and schedule of treatment (daily i.v. injection of 20 μg for 5 days) may not be the optimal and/or the tumor model may not be ideal for this particular study. In addition, we found that treatment with anti-HER2/*neu* IgG3-(GM-CSF) increases the endogenous humoral immune response against the human HER2/*neu* (39). Since we have evidence that endogenous antibodies may inhibit the binding of recombinant anti-HER2/*neu* IgG3 to the tumor cells (39), this enhanced antibody response in anti-HER2/*neu* IgG3-(GM-CSF) treated mice may further interfere with the binding of the anti-HER2/*neu* IgG3-(GM-CSF) to the cancer cells resulting in less effective anti-tumor activity. However, we would like to stress that this may be a limitation only in the studies using murine tumors in which the expression of HER2/*neu* is not related to cell survival. In patients, the ability of anti-HER2/*neu* IgG3-(GM-CSF) to elicit a strong humoral immune response may be

advantageous since antibodies targeting HER2/*neu* on human tumors appear to directly inhibit their growth (15). Therefore increasing the immune response using cytokines such as GM-CSF may facilitate tumor eradication. In fact, immunization using GM-CSF fused to the immunoglobulin expressed by a lymphoma can cause regression of the lymphoma in mice(61). The dramatically increased antibody response to the TAA HER2/*neu* is consistent with effective tumor targeting by anti-HER2/*neu* IgG3-(GM-CSF).

The isotype of the humoral immune response against human IgG and human HER2/*neu* suggests that anti-HER2/*neu* IgG3-(GM-CSF) has the ability to enhance both Th1 (T cell-directed) and Th2 (B cell-directed) immune responses. However, we do not know the effector mechanism responsible for the anti-tumor activity of anti-HER2/*neu* IgG3-(GM-CSF) observed in animals bearing CT26-HER2/*neu* tumors. Although ADCC mediated by effector cells such as macrophages, eosinophils, and NK cells is a possibility, $CD8^+$ (27) and $CD4^+$ (27, 30) cells may also play a role as they have been shown to be necessary for protection against tumor cell challenge in mice vaccinated with irradiated GM-CSF secreting tumor cells.

In conclusion, our results suggest that an anti-HER2/*neu* IgG3-(GM-CSF) fusion protein containing human GM-CSF may be effective in patients with tumors overexpressing HER2/*neu*. The combination of an anti-HER2/*neu* antibody with GM-CSF yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down regulation of HER2/*neu* expression, ADCC and the stimulation of a strong anti-tumor immune response through the immunostimulatory activity of GM-CSF. In addition, the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein

may be effective against tumor cells which express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* antibody therapy (14). Because of GM-CSF's ability to elicit an immune response to associated antigens, it is also possible that association of anti-HER2/*neu* IgG3-(GM-CSF) with soluble ECD^{HER2} shed by tumor cells will enhance the anti-tumor immune response.

Finally we would like to stress that anti-HER2/*neu* IgG3-(GM-CSF) would not be a replacement for Herceptin, but instead would provide an alternative therapy to be used in combination with the antibody or other anti-cancer approaches. These approaches might include chemotherapy or other anti-HER2/*neu* antibody fusion proteins such as anti-HER2/*neu* with the costimulator B7.1 (40) or the cytokine IL-12 (46). The availability of more than one antibody fusion protein will allow us to explore potential synergistic effects that may be obtained from manipulating the immune response.

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Footnotes

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[3] The abbreviations used in this paper are: TAA, tumor-associated antigen; GM-CSF granulocyte/macrophage colony-stimulating, IMDM, Iscove's modification of Dulbecco's medium; DNS, N,N dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); rmGM-CSF, recombinant murine GM-CSF; FPLC, fast protein liquid chromatography; ECD^{HER2}, extracellular domain of HER2/*neu* antigen; AP, akaline phosphatase.

Table I. *Mean Tumor Volumes and Statistical Significance.*

Days after	Mean Tumor Volumes [a]			Significance[b]	
the Challenge	PBS	IgG3	IgG3-(GM-CSF)	(*p*) 1	(*p*) 2
6	60.8	71	37.6	0.02	0.0006
9	211	224.5	110.5	0.0008	0.0003
12	578.2	631.8	264.9	0.0001	0.0001
15	1041.8	1155.6	655.3	0.0053	0.0002

[a] 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15 and the volume was calculated for each mouse of each treatment group. The experiment was made twice under identical conditions. Mean Tumor Volumes respresents the average tumor volume for each treatment group when the data of the two experiments were pooled.

[b] Statistical analysis of the anti-tumor experiments was done using a two-tailed Student's *t*-test. For all cases results were regarded significant if p values were ≤ 0.05. (p) 1 and (p) 2 represent the p obtained when Mean Tumor Volumes of the group injected with anti-HER2/*neu* IgG3-(GM-CSF) were compared with PBS and anti-HER2/*neu* IgG3 controls respectively.

Table II. *Murine anti-human HER2/neu and anti-human IgG3 titers* [a].

	anti-HER2/*neu* titers			anti-human IgG titers		
Mouse No.	PBS	IgG3	IgG3-(GM-CSF)	PBS	IgG3	IgG3-(GM-CSF)
1	150	150	12150	N/A[b]	450	4050
2	12150	450	4050	N/A	450	36450
3	450	450	1350	N/A	150	4050
4	450	450	1350	N/A	150	4050
5	1350	450	4050	N/A	450	12150
6	450	450	450	N/A	150	4050
7	50	150	4050	N/A	150	1350
8	450	450	1350	N/A	150	4050

[a] Groups of 8 mice injected s.c. with 10^6 CT26-HER2/*neu* cells were treated beginning the next day with five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Mice were bled 15 days after the injection of the tumor cells and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or human IgG3. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm) after 1 hr of incubation.

[b] Not applicable.

Figure Legends

Fig 1. Schematic diagram of the construction and expression of anti-HER2/*neu* IgG3-(GM-CSF). The expression vector for anti-HER2/*neu* IgG3-(GM-CSF), pAH1792, was constructed by 3 way ligation of the fragments containing the V_H anti-HER2/*neu* and constant IgG3 regions from pAT6611, the expression vector backbone from pAH4874, and GM-CSF from pAT1791. A solid line outside the plasmid indicates the fragment used in the three-way ligation. TAOL 5.2.3, a transfectant of P3X63Ag8.653 expressing a light chain with the anti-HER2/*neu* variable region was used as a recipient for transfection of the anti-HER2/*neu* IgG3-(GM-CSF) heavy chain expression vector pAH1792.

Fig 2. SDS-PAGE analysis of anti-HER2/*neu* IgG3-(GM-CSF). Secreted anti-HER2/*neu* IgG3-(GM-CSF) was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow and analyzed by SDS-PAGE under non-reducing (A) and reducing (B) conditions. Included for comparison is anti-HER2/*neu* IgG3 without attached GM-CSF. The positions of the m.w. standards are indicated at the left side.

Fig 3. Flow cytometry demonstrating the specificity of anti-HER2/*neu* IgG3-(GM-CSF) for the HER2/*neu* expressed on the surface of CT26-HER2/*neu*. CT26-HER2/*neu* (panels A, B and C) or the non-HER2/*neu* expressing parental cell line CT26 (panels D, E and F) were stained with anti-DNS human IgG3 (panels A and D), anti-HER2/*neu* human IgG3 (panels B and E) or anti-HER2/*neu* IgG3-(GM-CSF) (panels C and F), followed by biotinylated goat anti-human IgG and PE-labeled streptavidin.

Fig 4. Bioactivity Assay. FDC-P1 cells were incubated with various concentrations of rmGM-CSF (□) or anti-HER2/*neu* IgG3-(GM-CSF) (Δ). The concentration of anti-HER2/*neu* IgG3-(GM-CSF) was adjusted to the GM-CSF portion of the fusion protein obtaining equivalent molar concentrations of rmGM-CSF and anti-HER2/*neu* IgG3-(GM-CSF). Proliferation was measured by a colorimetric assay and read at 490 nm. All results are expressed as mean OD_{490} of quadruplicate wells with a standard deviation less than 20 % for each concentration.

Fig. 5. Macrophage mediated cytotoxicity. A: 1 x 10^4 ^{3}H-labeled CT26-HER2/*neu* target cells were cultured for 24 hrs with anti-HER2/*neu* IgG3 (5 μg /mL), the equivalent molar concentration of anti-HER2/*neu* IgG3-(GM-CSF) or medium alone in the presence of J774.2 macrophage effector cells an effector:target ratio of 10. B: Effector cells were pre-incubated for 24 hrs with anti-HER2/*neu* IgG3-(GM-CSF) (6.72 x 10^{-2} μg/ml), the equivalent molar concentration of anti-HER2/*neu* IgG3 or medium alone, washed and then incubated with 1 x 10^4 ^{3}H-labeled CT26-HER2/*neu* target cells for 24 hrs. For both assays intact DNA from live target cells was collected by a cell harvester and radioactiviy was measured using a scintillation counter. Bars represent the standard deviation of quadruplicate samples.

Fig 6. Half-life of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. Groups of 2 mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) of ^{125}I-labeled anti-

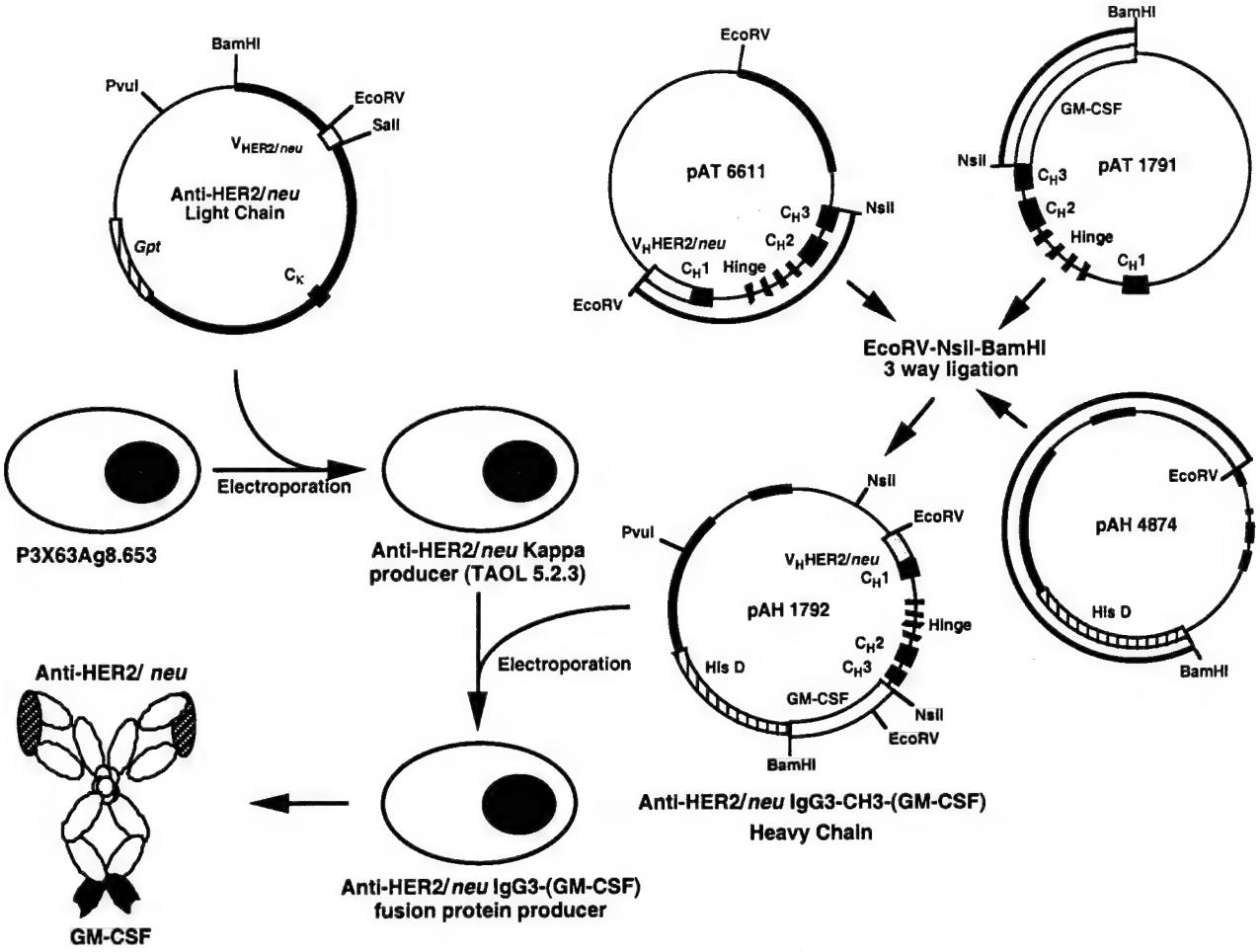
HER2/*neu* IgG3 (△), anti-HER2/*neu* IgG3-(GM-CSF) (○), or 1 μCi (0.5 μg) of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) mixed with 20 μg of cold anti-HER2/*neu* IgG3-(GM-CSF) (□). At various intervals after injection of the ^{125}I-labeled protein residual radioactivity was measured using a mouse whole body counter. The results represent the mean of 2 mice. The error bars represent the range of values obtained.

Fig 7. Biodistribution of anti-HER2/*neu* IgG3-(GM-CSF). Two groups of 4 mice were injected i.v. via the lateral tail vein with 1 μCi (0.5 μg) of ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF) and mice were euthanized after 4 hrs, which is the equivalent of two half-lives for the injected dose or after 16 hrs. Various organs and blood were collected, weighed and radioactivity measured using a gamma counter. Data are presented as percent of injected dose per gram of tissue (%ID/g tissue). Bars represent the standard deviation of the data obtained.

Fig 8. Tumor targeting of anti-HER2/*neu* IgG3-(GM-CSF). 10^6 CT26-HER2/*neu* and CT26 cells were injected separately into the right and left flanks, of 3 BALB/c mice. One week later when the tumor diameter was approximately 1.0 cm, groups of 3 mice were injected i.v. via the lateral tail vein with ^{125}I-labeled anti-HER2/*neu* IgG3-(GM-CSF). Mice were euthanized 12 hrs after injection. Blood and tumors were collected, weighed and radioactivity measured by a gamma counter. Data are presented as percent of injected dose per gram of tumor (%ID/g tumor).

Fig 9. Anti-Tumor Activity of anti-HER2/*neu* IgG3-(GM-CSF) and anti-HER2/*neu* IgG3. 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(GM-CSF), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15. The volume was calculated for each mouse of each treatment group, PBS (panel A), anti-HER2/*neu* IgG3 (panel B), and anti-HER2/*neu* IgG3-(GM-CSF) (panel C). Experiments 1 and 2 were made under identical conditions but at different time.

Fig 10. Isotype profile of antibodies specific for HER2/*neu* and human IgG3. Pooled sera from mice treated with PBS (solid bar), anti-HER2/*neu* IgG3 (checkered bar), or anti-HER2/*neu* IgG3-(GM-CSF) (clear bar) were analyzed by ELISA for antibodies of different isotypes recognizing either anti-HER2/*neu* IgG3 (A) or ECD^{HER2} (B).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PvuI
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His D
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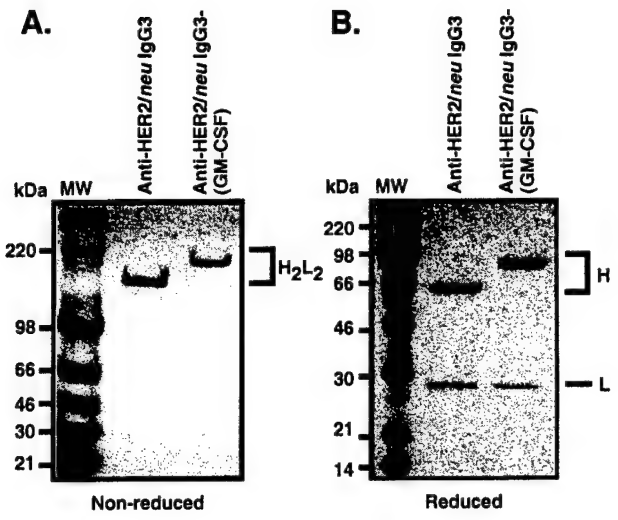
fusion protein producer
GM-CSF

Figure 1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B.
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Figure 2

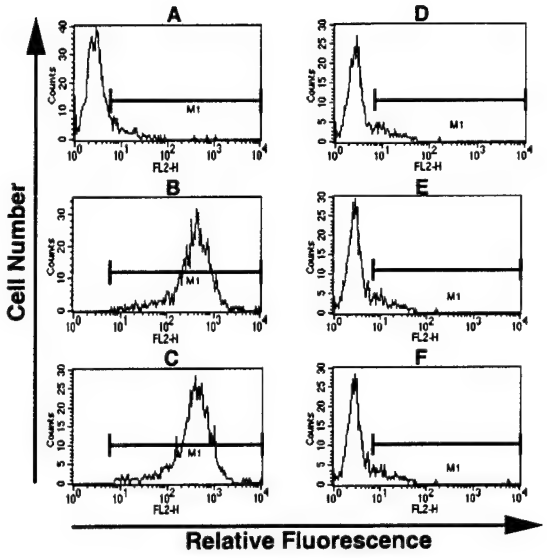


Figure 3

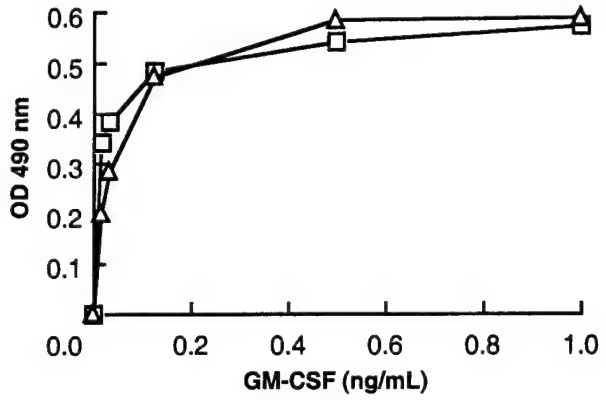
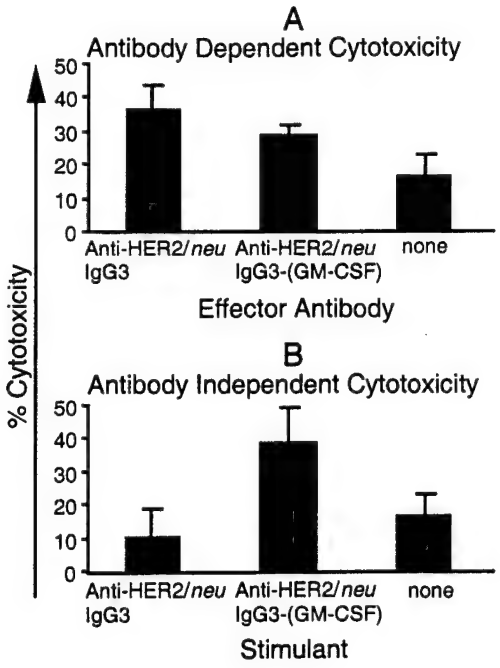


Figure 4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Antibody Independent Cytotoxicity
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Anti-HER2/neu IgG3-(GM-CSF)
none
Stimulant

Figure 5

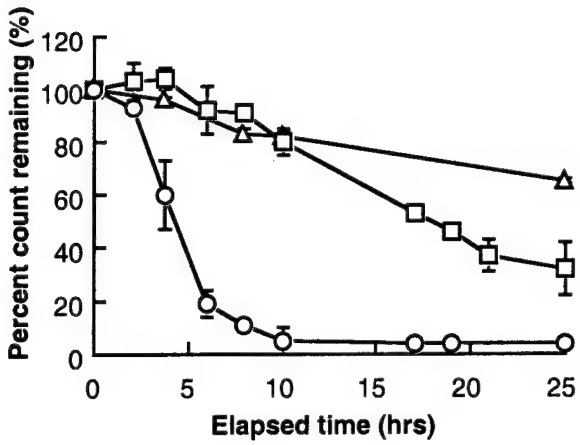
Percent count remaining (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
Elapsed time (hrs)

Figure 6

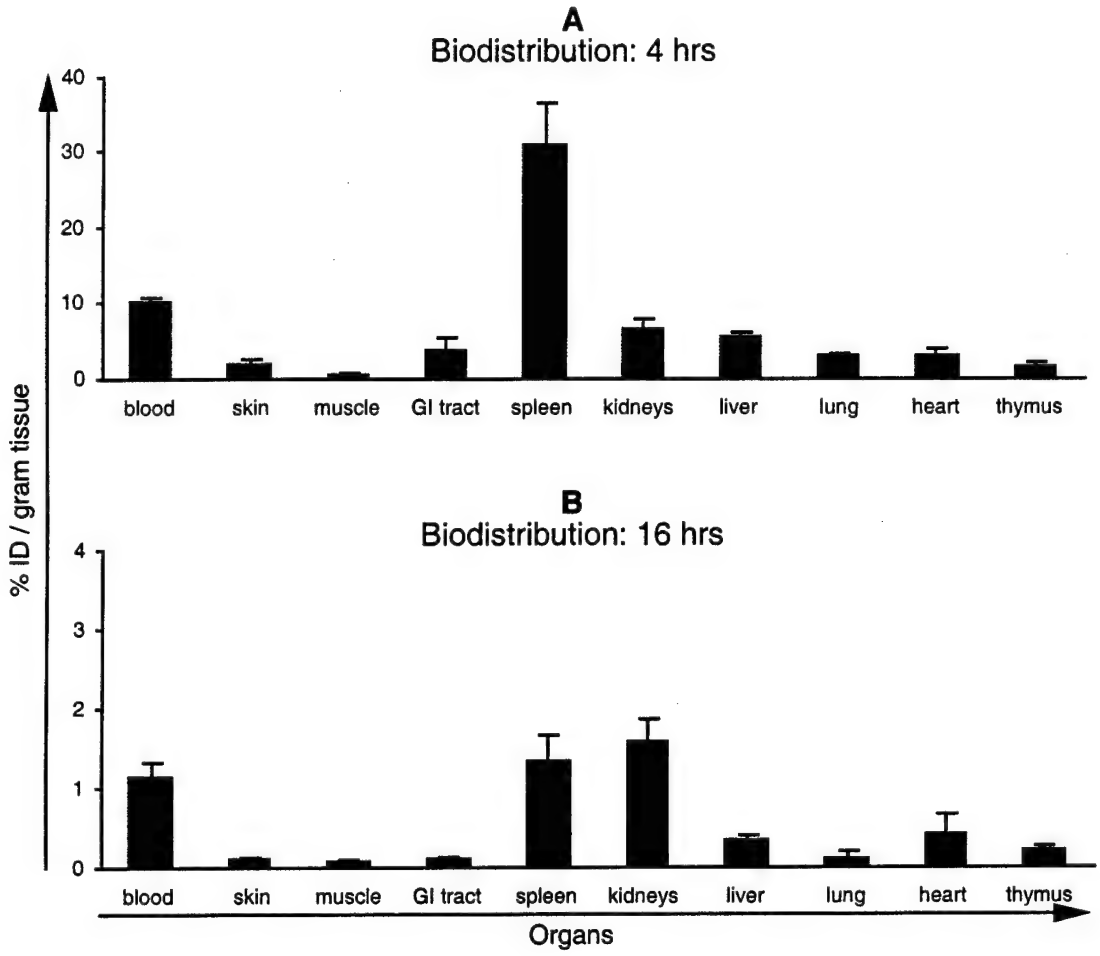
A
Biodistribution: 4 hrs
40
30
20
10
0
blood
skin
muscle
GI tract
spleen
kidneys
liver
lung
heart
thymus
B
Biodistribution: 16 hrs
4
3
2
1
0
% ID / gram tissue
blood
skin
muscle
GI tract
spleen
kidneys
liver
lung
heart
thymus
Organs

Figure 7

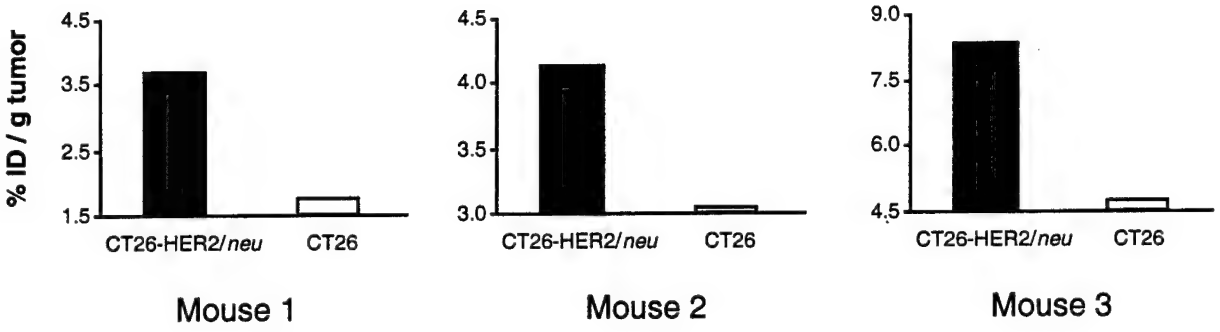
% ID / g tumor
4.5
3.5
2.5
1.5
CT26-HER2/neu
CT26
Mouse 1
4.5
4.0
3.5
3.0
CT26-HER2/neu
CT26
Mouse 2
9.0
7.5
6.0
4.5
CT26-HER2/neu
CT26
Mouse 3

Figure 8

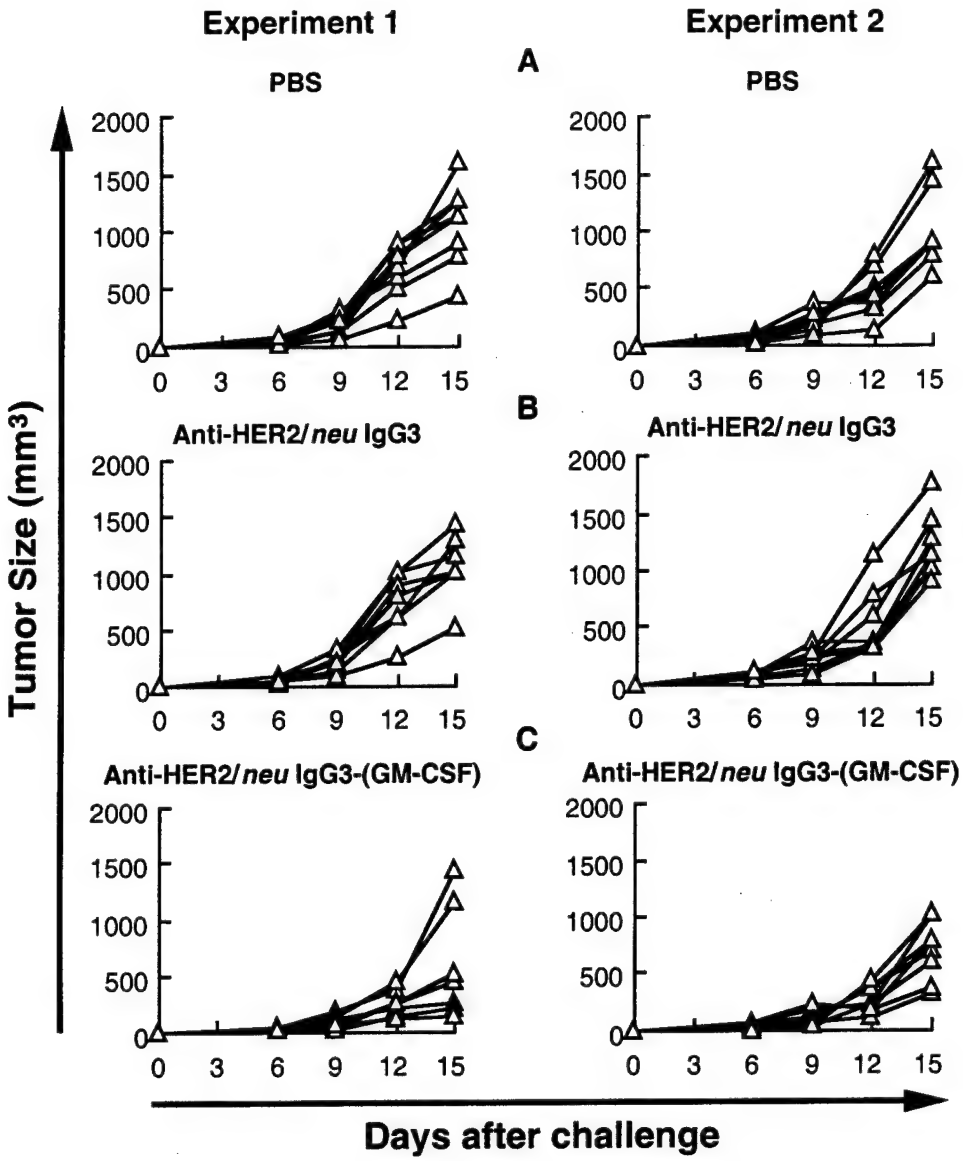
Experiment 1
Experiment 2
A
PBS
PBS
B
Anti-HER2/neu IgG3
Anti-HER2/neu IgG3
C
Anti-HER2/neu IgG3-(GM-CSF)
Anti-HER2/neu IgG3-(GM-CSF)
Tumor Size (mm3)
2000
1500
1000
500
0
0 3 6 9 12 15
Days after challenge

Figure 9

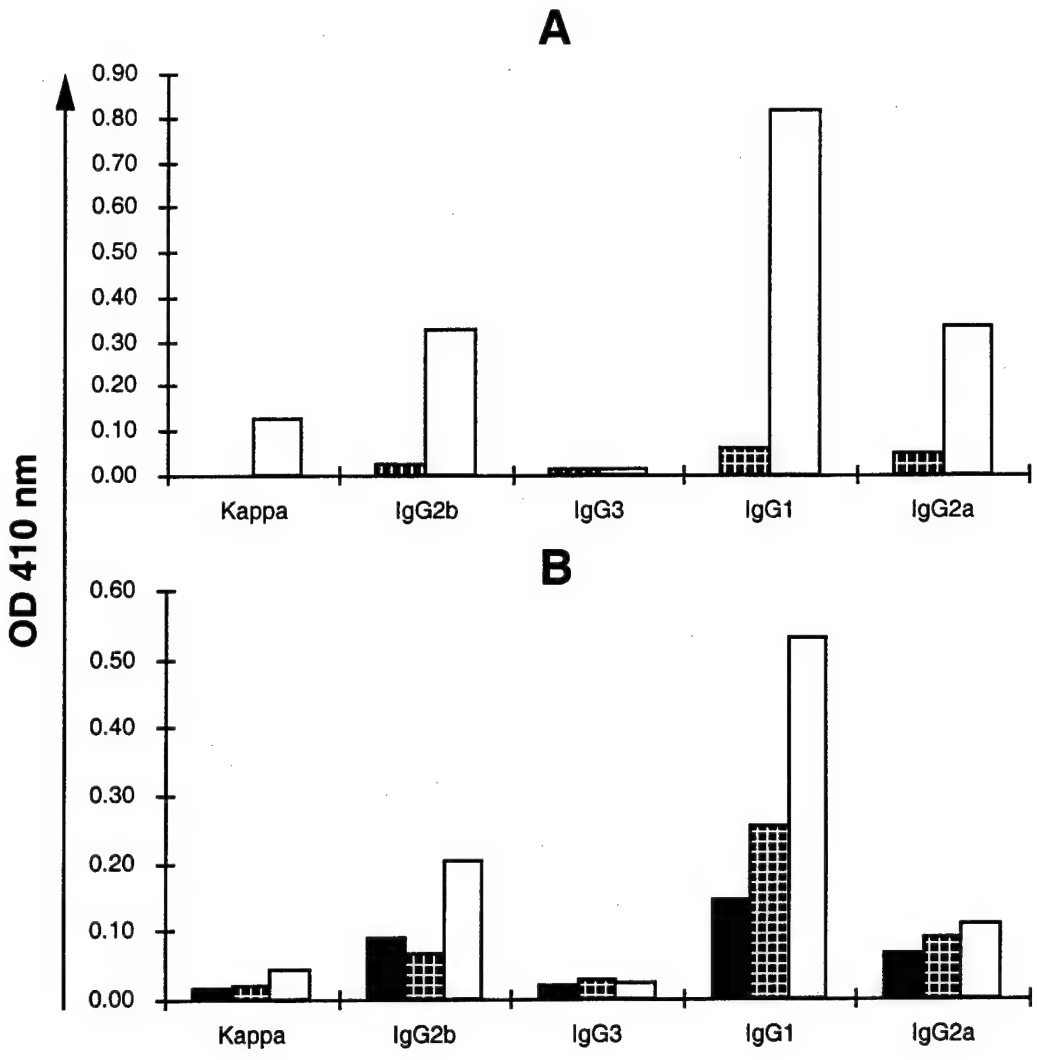
A
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
OD 410 nm
Kappa
IgG2b
IgG3
IgG1
IgG2a
B
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Kappa
IgG2b
IgG3
IgG1
IgG2a

Figure 10

A Single-Chain IL-12 IgG3 Antibody Fusion Protein Retains Antibody Specificity and IL-12 Bioactivity and Demonstrates Antitumor Activity[1]

Lisan S. Peng, Manuel L. Penichet, and Sherie L. Morrison[2]

IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antimetastatic effects. However, systemic administration of IL-12 can be toxic. Tumor-specific Abs provide a means to selectively target a metastatic/residual nodule and deliver therapeutic quantities of an immunostimulatory molecule like IL-12 with lower systemic levels and ideally, toxicity. We report the construction and characterization of an Ab fusion protein in which single-chain murine IL-12 is fused to an anti-Her2/*neu* Ab at the amino terminus (mscIL-12.her2.IgG3). The use of single-chain IL-12 in the fusion protein simplifies vector construction, ensures equimolar concentrations of the two IL-12 subunits, and may confer greater stability to the fusion protein. SDS-PAGE analysis shows this 320-kDa protein is secreted and correctly assembled. FACS analysis demonstrates that this fusion protein binds to cells transfected with the Her2/*neu* Ag, thus retaining Ab specificity; this fusion protein also binds to a cell line and to PHA-activated PBMC that express the IL-12R, thus demonstrating cytokine receptor specificity. T cell proliferation assays and NK cytotoxicity assays demonstrate that this fusion protein exhibits IL-12 bioactivity comparable to recombinant murine IL-12. In vivo studies demonstrate that this fusion protein has antitumor activity. These results are significant and suggest that this IL-12 Ab fusion protein can effectively combine the therapeutic potential of IL-12 with the tumor-targeting ability of the Ab and may provide a viable alternative to systemic administration of IL-12. ***The Journal of Immunology*****, 1999, 163: 250–258.**

The management of residual and metastatic disease is a central problem in the treatment of cancer. Chemotherapeutic strategies can be effective, but are frequently limited by various toxicities. Therefore, additional modalities are needed to achieve disease containment or elimination. One approach has been to attempt to elicit a specific immune response by the host against tumor-associated Ags. Treatment with cytokines has been shown to render some nonimmunogenic tumors immunogenic, activating a protective immune response (1–4). However, when cytokines are given systemically there are frequently problems with toxicity that make it impossible to achieve an effective dose at the site of the tumor (5, 6). Ideally, strategies which increase the cytokine concentration at the site of the tumor and allow for lower systemic levels should be more effective.

Two approaches to achieving high levels of cytokine at the site of the tumor have been direct injection of cytokine into the tumor or transfer of the gene encoding the cytokine into tumor cells (7). While both methods have been shown to be effective, they also have significant limitations: direct injection into micrometastases is not possible, and currently gene transfer involves ex vivo manipulation of tumor cells, which makes treatment of large numbers of patients difficult and costly. Abs provide an alternative specific delivery vehicle in which tumor-specific Abs can be used to selectively target a metastatic/residual nodule and deliver an immunostimulatory molecule like a cytokine. The specific targeting should make it possible to elicit a systemic tumor-specific immune response without accompanying systemic toxicity.

There are many different types of tumor-associated Ags: oncofetal Ags (e.g., carcinoembryonic Ag (8)), Ags expressed on cells at a particular stage of differentiation (e.g., IL-2R (9)), growth factor receptors (e.g., transferrin receptor (10)), oncogene products (e.g., *c-myc* (11)), and the Id expressed by the surface Ig of lymphoma cells (12). These tumor-associated Ags distinguish normal from tumor tissue and have been used as targets for cancer therapy (13–17). Her2/*neu*, also known as *c-erbB*-2, is a cell surface oncogene product that is amplified and/or overexpressed in 25–30% of human breast and ovarian cancers with this overexpression associated with poor prognosis (18, 19). Humanized anti-Her2/*neu* has been shown to be an effective therapeutic agent in clinical trials (20). These trials demonstrate that metastatic breast cancer can be effectively targeted through the Her2/*neu* Ag and suggest that Abs specific for Her2/*neu* would be effective vehicles for targeting cytokines to the sites of the tumors.

Several different cytokines are attractive candidates for enhancing tumor-specific immune responses. IL-2 induces the proliferation of T cells, supports the growth of Ag-specific T cell clones, and enhances the activity of T and NK cells (21). Fusion of IL-2 to Abs specific for tumor-associated Ags such as ganglioside GD2 and the Id of a murine lymphoma has resulted in fusion proteins that have shown much promise as agents for stimulating tumor-specific immune responses (22–27). Indeed, an IL-2-Ab fusion protein specific for GD2 was able to generate an immune response

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0022-1767/99/$02.00

that eliminated metastatic disease in a murine model of melanoma (22).

Another cytokine that has great potential for use in tumor immunotherapy is IL-12. IL-12 is a heterodimeric cytokine with many actions on innate and cellular immunity that may have antitumor and antimetastatic effects. IL-12 can activate T and NK cells, induce the production of IFN-γ, and stimulate naive $CD4^+$ T cells to differentiate toward the Th1 phenotype (28, 29). A Th1 response involves the secretion of a cytokine profile that activates cytotoxic T cells and macrophages, which could be desirable in an antitumor immune response. In addition, IL-12 may act through nitric oxide to cause cell-cycle arrest of tumor cells (4), and through induction of inducible protein-10 to inhibit angiogenesis (30).

Bioactive IL-12 requires the expression of two separate genes, p40 and p35, and correct heterodimer assembly (31). To address this issue, Gillies et al. have recently reported the construction of an Ab-IL-12 fusion protein in which the p35 subunit was fused to the carboxyl terminus of an Ab; the p40 subunit was expressed as a separate polypeptide that must then assemble with the p35 subunit. Although this IL-12/Ab fusion protein was functional, the IL-12 bioactivity was 2-fold lower than rIL-12 (32). An alternative that eliminates the need to assemble two independently produced peptides is to express IL-12 as a single chain with the p40 and p35 subunits joined by a flexible linker. We have now used this alternative approach and constructed an Ab fusion protein in which murine single-chain (msc)[3] IL-12 (p40.linker.p35) is fused to an anti-Her2/*neu* Ab at the amino terminus of the H chain (mscIL-12.her2.IgG3). Importantly, this fusion protein retains Ab specificity, exhibits IL-12 bioactivity comparable to recombinant murine (m) IL-12, and demonstrates antitumor activity in vivo.

Materials and Methods

Cell lines and reagents

P3X63Ag8.653 cells (American Type Culture Collection, Manassas, VA), CT26 cells (murine colon adenocarcinoma cells kindly provided by Young Chul Sung, Pohang University, Korea), and CT26/Her2 cells (developed in our laboratory by transfection of CT26 cells with the cDNA encoding Her2/*neu* using methods previously described (33)) were cultured in IMDM supplemented with 5% bovine calf serum, L-glutamine, penicillin, and streptomycin. K562 cells (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% FBS, sodium pyruvate, HEPES, and D-glucose. Kit255/K6 cells (kindly provided by Jim Johnston, DNAX, Palo Alto, CA) were maintained in RPMI 1640 supplemented with 10% FBS and 100 IU recombinant human (h) IL-2/ml (kindly provided by Chiron, Emeryville, CA). rmIL-12 reference standard was kindly provided by Stanley Wolf (Genetics Institute, Cambridge, MA).

Mice

Female 6- to 8-wk-old BALB/c mice were obtained from Taconic Farms (Germantown, NY) and conventionally housed. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Ab expression vectors

her2.IgG3. The variable L and H chain domains were obtained from the plasmid pAK19 containing the humanized humAb4D5–8 Ab (generously provided by Paul Carter, Genentech, South San Francisco, CA) (34, 35) and cloned as previously described (36) into mammalian expression vectors for human κ L chain and IgG3 H chain, respectively.

mscIL-12.her2.IgG3. The cDNA for mscIL-12 (p40 subunit linked by a $(Gly_4Ser)_3$ flexible linker to the p35 subunit from which the first 22 aa (leader sequence) were deleted) was generously provided by Richard Mulligan (Harvard Medical School, Boston, MA) as plasmid pSP72.mIL-12.p40.linker.Δp35. mscIL-12 was amplified from the plasmid by PCR using the sense primer 5′-CCCCAAGCTTGATATCCACCATGGGTC CTCAGAAGCTAACC-3′ and the antisense primer 5′-CCCGAATTCGT TAACCGGCGGAGCTCAGATAGCCC-3′. The PCR product was cloned as a *Hin*dIII/*Hpa*I fragment to the 5′ end of a cassette encoding the $(Gly_4Ser)_3$ linker sequence of Huston et al. (37) fused to the anti-Her2/*neu* V_H sequence. The resulting mscIL-12.linker.V_H coding sequences were excised as an *Eco*RV/*Nhe*I fragment and cloned into an expression vector for human IgG3 H chain (38).

Recombinant Ab expression, immune precipitation, and purification

Transfection, expression, and purification of the recombinant Abs were performed as previously described (39) to obtain mscIL-12.her2.IgG3. Briefly, 1×10^7 P3X63Ag8.653 myeloma cells were transfected by electroporation with 10 μg of each of the mscIL-12.her2.IgG3 H and anti-Her2 κ L chain expression vectors (linearized with *Pvu*I). Transfected cells were plated at 2×10^4 cells/well in a flat-bottom 96-well tissue culture plate and selected with the addition of 10 mM histidinol (Sigma, St. Louis, MO) on days 3 and 5 after transfection. Wells were screened for Ab secretion after 10–14 days by ELISA using 96-well flat-bottom plates coated with goat anti-human IgG (Zymed, South San Francisco, CA). Supernatant from the transfected cells was applied, followed by the addition of goat anti-human κ conjugated with alkaline phosphatase (Sigma). Binding was detected by the addition of phosphatase substrate (*para*-nitrophenyl phosphate, disodium; Sigma), and positive wells were expanded.

To determine the size and assembly pattern of the secreted recombinant mscIL-12.her2.IgG3 Ab, supernatants from cells grown overnight in medium containing [^{35}S]methionine (Amersham, Piscataway, NJ) were immunoprecipitated with polyclonal rabbit anti-human IgG and rabbit anti-human κ (produced by Letitia A. Wims in our laboratory), followed by staphylococcal protein A (IgGSorb; The Enzyme Center, Malden, MA). Precipitated Abs were analyzed on SDS-polyacrylamide gels in the presence or absence of reducing agent (2-ME).

For the purification of mscIL-12.her2.IgG3, high producing clones were expanded in roller bottles in IMDM plus 1% fetal clone serum plus Glutamax (Life Technologies, Rockville, MD), and cell-free culture supernatant was collected. Culture supernatants were passed through a protein A column, the column was washed with 10 ml PBS, and the proteins were successively eluted with 2 ml of 1 M citric acid, pH 4.5, 5 ml of 0.1 M glycine, pH 2.5, and 2 ml of 0.1 M glycine, pH 2.0. The eluted fractions were neutralized immediately with 2 M Tris-HCl, pH 8.0. The fractions were concentrated using Ultra-free-15 filters (Millipore, Bedford, MA) with a cut-off of 100 kDa and dialyzed. Using this method, 2 L of culture supernatant yields ~0.8 mg mscIL-12.her2.IgG3.

*Assays of binding to Her2/*neu *Ag and IL-12R*

Ag binding. CT26 or CT26/Her 2 were incubated with mscIL-12.her2.IgG3, her2.IgG3, or dansyl.IgG3 (an IgG3 isotype control Ab specific for the hapten dansyl) for 1 h at 4°C. The cells were washed and incubated 2 h at 4°C with PE-labeled goat anti-human IgG (PharMingen, San Diego, CA) and analyzed by flow cytometry. Analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Persistence of Ab binding at the cell surface. CT26/Her2 cells were incubated with mscIL-12.her2.IgG3, her2.IgG3, or dansyl.IgG3. The cells were washed and incubated at 37°C in culture medium. At different time points (0, 1, 4, and 24 h), an aliquot of cells was removed and stained with PE-conjugated anti-human IgG for FACS analysis. The mean fluorescence was calculated as a percentage of the maximum mean fluorescence at time zero.

Binding to IL-12R. Kit225/K6 cells, a subclone of the human T leukemic cell line that expresses the IL-12R (40), were incubated with her2.IgG3 or mscIL-12.her2.IgG3. Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis. In a second assay, PHA-activated PBMC were incubated with her2.IgG3 or mscIL-12.her2.IgG3. PBMC have been shown to express IL-12Rs following activation with PHA and IL-2 (41). Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis.

Proliferation assays

Proliferation assays were performed as previously described (42). PBMC were isolated from normal blood donors by Ficoll-Hypaque density centrifugation (Ficoll-Paque, Pharmacia, Piscataway, NJ). These cells were then depleted of monocytes by plastic adherence, and nonadherent cells

[3] Abbreviations used in this paper: msc, murine single-chain; m, murine; h, human; sc, single-chain; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PMS, phenazine methosulfate; PI, propidium iodide.

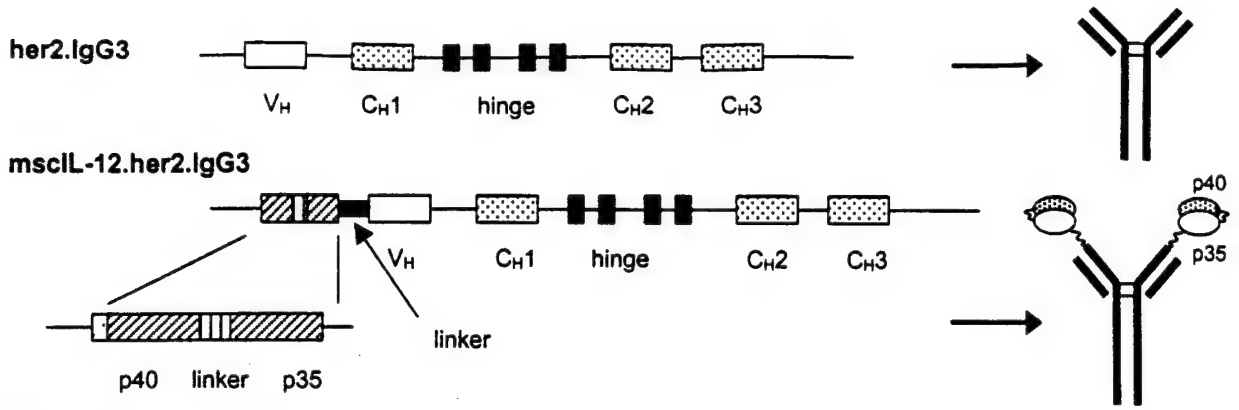

FIGURE 1. Structure of her2.IgG3 and mscIL-12.her2.IgG3. The construction of vectors for the expression of her2.IgG3 was previously described (36). For the construction of mscIL-12.her2.IgG3, mscIL-12 was amplified from the plasmid pSP72.mIL-12.p40.linker.Δp35 (kindly provided by Richard Mulligan) and joined to a $(Gly_4Ser)_3$ linker located at the amino terminus of the V_H region of the her2.IgG3 Ab.

were resuspended at 5×10^5 cells/ml in supplemented medium [1:1 complete RPMI 1640:complete DMEM plus 5% human AB serum (Irvine Scientific, Santa Ana, CA), 10 mM HEPES, 0.006% (w/v) L-arginine monohydride, and 0.1% (w/v) dextrose] containing 2 μg/ml PHA-P (Difco Laboratories, Detroit, MI) and were cultured for 3 days. Cells were then split 1:1 with fresh supplemented medium containing 20 IU/ml rhIL-2 (kindly provided by Chiron Corporation) and incubated for a further 24–48 h. The PHA blasts were then washed with acidified RPMI 1640, pH 6.4, and rested in RPMI 1640 plus 0.5% human AB serum for 3–4 h. The cell concentration was adjusted to 2×10^6 cells/ml in supplemented media. Neutralizing anti-IL-2 Ab (BioSource International, Camarillo, CA) was added at 1 μg/ml to block IL-2-induced proliferation.

Serial 1:3 dilutions of equivalent protein concentrations of mIL-12, mscIL-12.her2.IgG3, and her2.IgG3 were made in supplemented medium over a range of 36 ng/ml to 16 pg/ml. Next, 50 μL cell suspension was mixed with 50 μL mIL-12, mscIL-12.her2.IgG3, her2.IgG3, or supplemented medium in triplicate in a flat-bottom 96-well tissue culture plate. After 48 h of culture at 37°C, 5% CO_2, proliferation was measured by the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)/phenazine methosulfate (PMS) assay (Promega, Madison, WI), and plates were read at OD_{490}.

Enhanced NK activity of PBMC

These assays were performed according to the methods of Hatam et al. (43) with modifications. Briefly, effector PBMC were isolated as described above, then resuspended in RPMI 1640 plus 10% FBS at $1–2 \times 10^6$ cells/ml. rmIL-12 at 5 ng/ml, an equivalent IL-12 concentration of mscIL-12.her2.IgG3, or an equivalent Ab concentration of her2.IgG3 were added to the PBMC and incubated for 16–18 h at 37°C, 5% CO_2. The cell concentration was then adjusted to $0.5–1 \times 10^6$ cells/ml. Target K562 cells (2×10^7) were washed two times with serum-free RPMI 1640, then resuspended in 1 ml Diluent C (Sigma). Then, 4 μM PKH67 was prepared by diluting the stock solution (Sigma) in Diluent C. The cell suspension and dye were mixed in equal volumes (1 ml each) in a polypropylene tube and incubated at room temperature for 2 min. An equal volume (2 ml) of FBS was added to stop the labeling reaction. The cells were washed three times with RPMI 1640 plus 10% FBS and resuspended at $1–2 \times 10^5$ cells/ml in RPMI 1640 plus 10% FBS.

For the NK cytotoxicity assay, 100 μL effector PBMC and 100 μL PKH67-labeled K562 cells were added to polystyrene 12 × 75 mm tubes to create E:T ratios of 50:1 and 100:1 and were incubated for 4 h at 37°C, 5% CO_2. At the end of the incubation, 0.5 ml isotonic propidium iodide (PI) at 5 μg/ml (Sigma) was added to each tube and immediately analyzed by FACS. Spontaneous cell death was determined by incubating either targets or effectors alone.

FACS analysis was performed with a FACScan (Becton Dickinson) equipped with a blue laser excitation of 15 mW at 488 nm. The two fluorochromes, PKH67 and PI, were electronically compensated using PKH67-labeled targets alone and unstained target cells whose membrane had been permeabilized by treatment with 0.1% Tween-20 in PBS for 10 min at 37°C. These cells were then washed twice and 0.5 ml of isotonic PI added before FACS analysis. Data were collected in list mode and analyzed using Cell Quest software (Becton Dickinson). At least 2000 target events were collected per sample. Percent cytotoxicity was calculated as (number of dead targets)/(total number of targets) × 100.

In vivo antitumor activity

A total of 1×10^6 CT26/Her2 cells in 0.15 ml PBS were injected s.c. into the right flank of syngeneic BALB/c mice on day 0. One group of mice was treated i.v. with mscIL-12.her2.IgG3 (at a concentration equivalent to 1 μg IL-12/day), her2.IgG3 (at a concentration equivalent to the Ab concentration of mscIL-12.her2.IgG3 administered/day), or PBS for 5 days beginning on day 1. A second group of mice was similarly treated beginning on day 6. In each group, 10 mice per treatment arm were used. Tumor growth was monitored and measured with a caliper every other day beginning on day 6 and continuing until day 20. At that point, all mice were euthanized and the tumors were harvested and weighed.

Results

Design and expression of single-chain (sc) IL-12 Ab fusion protein

The construction of her2.IgG3 and vectors for the production of H chain fusion proteins was previously described (36). For the present studies, we elected to use mIL-12 in our fusion protein because mIL-12 is biologically active on activated murine and human T and NK cells, while murine T and NK cells do not respond to hIL-12 (44). mscIL-12 was amplified from plasmid pSP72.mIL-12.p40.linker.Δp35 and cloned at the amino terminus of the V_H region of her2.IgG3 (Fig. 1). A flexible $(Gly_4Ser)_3$ linker was positioned between IL-12 and the V region to facilitate both correct folding of the Ab and IL-12 and simultaneous Ag and IL-12R binding. The mscIL-12.her2.IgG3 H chain and κ L chain were cotransfected into P3X63Ag8.653 myeloma cells, and stable transfectants secreting Ab were selected using the anti-human IgG ELISA described in *Materials and Methods*.

To determine the molecular mass and assembly of the secreted Ab, the cells were grown overnight in [^{35}S]methionine. Anti-human IgG and anti-human κ followed by insoluble protein A were added to the supernatant and cell lysates, and the immunoprecipitates were analyzed by SDS-PAGE. In the absence of reducing agents, IgG3 migrates with an apparent molecular mass of 170 kDa, while mscIL-12.her2.IgG3 is about 320 kDa, the expected molecular mass of the fusion protein (Fig. 2*A*). Following treatment with the reducing agent 2-ME, L chains migrating with an apparent molecular mass of ~25 kDa are seen for both proteins. However, the IgG3 has a H chain of ~60 kDa, while mscIL-12.her2.IgG3 has a H chain of ~135 kDa. Thus, proteins of the expected molecular mass are produced, and fusion of scIL-12 to

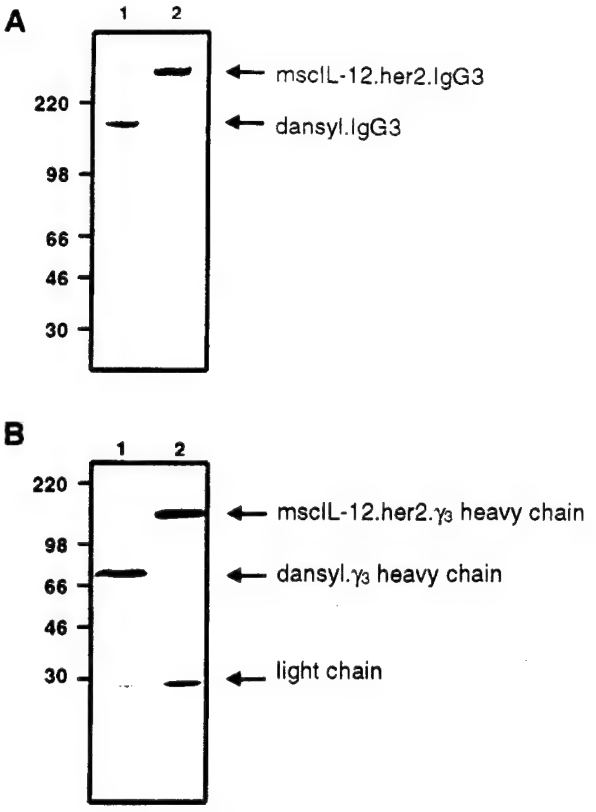

FIGURE 2. SDS-PAGE analysis. Cell lines expressing mscIL-12.her2.IgG3 or dansyl.IgG3 (isotype control) were grown overnight in [^{35}S]methionine. The supernatant and cell lysates were immunoprecipitated with anti-human IgG and anti-human κ and insoluble protein A and analyzed by SDS-PAGE in the absence (*A*) or presence (*B*) of 2-ME. The samples run in both panels were dansyl.IgG3 (*lane 1*) and mscIL-12.her2.IgG3 (*lane 2*). The positions of molecular mass markers are indicated at the left.

her2.IgG3 does not appear to alter the assembly and secretion of the H_2L_2 form of the Ab.

Ag binding and persistence of Ab binding at the cell surface

The ability of mscIL-12.her2.IgG3 to bind to the Her2/*neu* antigenic target was examined using flow cytometry. Both mscIL-12.her2.IgG3 (Fig. 3*B*) and her2.IgG3 (Fig. 3*C*) specifically bound to CT26/Her2; neither Ab bound to parental CT26 cells (data not shown). Importantly, the same fluorescence intensity was seen with both her2.IgG3 and mscIL-12.her2.IgG3, suggesting that both

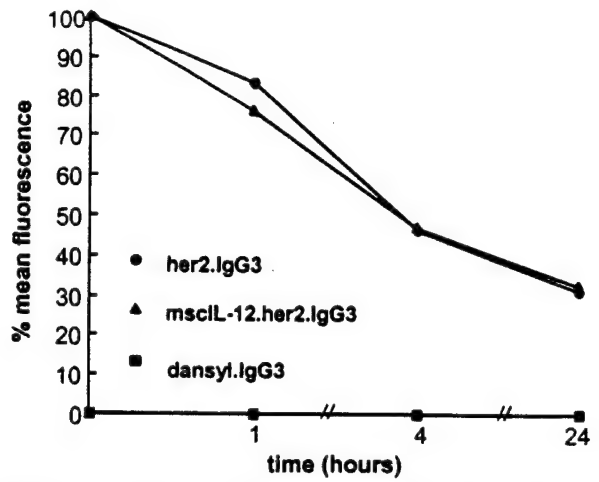

FIGURE 4. Stability of the recombinant her2.IgG3 and mscIL-12.her2.IgG3 on the surface of the murine tumor cell CT26 expressing human Her2/*neu* (CT26/her2). CT26/her2 cells were incubated with her2.IgG3, mscIL-12.her2.IgG3, or dansyl.IgG3 isotype control. The cells were washed and incubated at 37°C. Aliquots were removed at 0, 1, 4, or 24 h and analyzed by flow cytometry using PE-conjugated anti-human IgG. The mean fluorescence is calculated as a percentage of the maximum mean fluorescence observed at time zero.

have similar affinity for Her2/*neu*. A control IgG3 Ab specific for the hapten dansyl did not bind to CT26/Her2 (Fig. 3*A*). These data indicate that the fusion of a 75-kDa scIL-12 to the amino terminus of each H chain of her2.IgG3 does not interfere with the ability of the Ab to recognize the Her2/*neu* Ag.

There is no significant difference in the persistence of Ab bound to the cell surface between mscIL-12.her2.IgG3 and her2.IgG3, with both still showing similar fluorescence intensity at all time points and >30% staining at 24 h (Fig. 4). These results indicate that fusion of the Ab with IL-12 does not affect the dissociation rate, internalization, or degradation of mscIL-12.her2.IgG3 at the surface of the cell compared with her2.IgG3. This suggests that the IL-12 in our fusion protein will be present at the cell surface to activate T and NK cells.

Binding to the IL-12R

The ability of mscIL-12.her2.IgG3 to bind to the IL-12R was determined by flow cytometry of both transformed and normal human cells. The mscIL-12.her2.IgG3 bound to Kit225/K6, a subclone of the human T leukemic cell line that expresses the IL-12R (40), while her2.IgG3 did not (Fig. 5*A*). Neither her2.IgG3 or

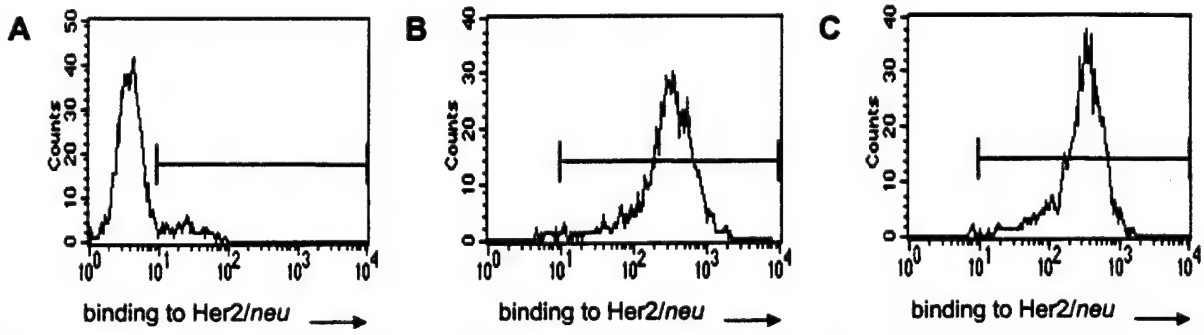

FIGURE 3. Ag binding. Flow cytometry demonstrating the reactivity of anti-Her2/*neu* Ab fusion proteins with Her2/*neu*. Murine CT26 cells transduced with the Her2/*neu* cDNA were incubated with either dansyl.IgG3 (isotype control) (*A*), mscIL-12.her2.IgG3 (*B*), or her2.IgG3 (*C*), followed by PE-labeled goat anti-human IgG.

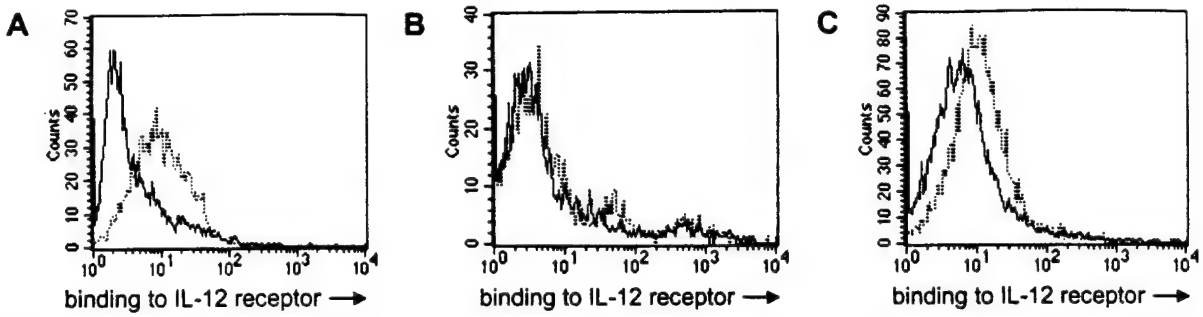

FIGURE 5. Binding to IL-12R. Kit225/K6 cells (*A*), which express IL-12Rs, resting PBMC (*B*), or PHA-activated PBMC (*C*) were incubated with her2.IgG3 (solid line) or mscIL-12.her2.IgG3 (dotted line). Binding was assayed by staining with PE-conjugated anti-human IgG followed by FACS analysis. Note that a shift in a peak means that the entire population of cells now reacts with the IL-12-bearing Ab.

mscIL-12.her2.IgG3 bound to the resting PBMC (Fig. 5*B*). The mscIL-12.her2.IgG3 bound to the PHA-activated PBMC while her2.IgG3 did not (Fig. 5*C*). These results show that the IL-12 in the fusion protein is able to bind to the IL-12R.

Proliferation assays

After establishing that mscIL-12.her2.IgG3 was correctly assembled, secreted, and retained the ability to bind both the Her2/*neu* Ag and the IL-12R, we investigated its biologic activity. All assays of IL-12 biological activity were expressed relative to the IL-12 concentration used (i.e., ng/ml). To obtain the IL-12 concentration of the fusion protein, the fraction of the IL-12-Ab fusion protein that was IL-12 (150 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein. In this way, the biological activity of rIL-12 and IL-12 in the fusion protein could be compared on a per molecule basis. Similarly, to ensure that equivalent Ab concentrations of her2.IgG3 and mscIL-12.her2.IgG3 were used, the fraction of the Ab-fusion protein that was Ab (170 kDa/320 kDa) was multiplied times the protein concentration of the fusion protein to obtain the Ab concentration of the fusion protein. The same concentration of her2.IgG3 was then used as a control.

One of the pleiotropic actions of IL-12 is the ability to induce the proliferation of PHA-activated lymphoblasts. We prepared PHA-activated PBMC and incubated them for 48 h with mIL-12, mscIL-12.her2.IgG3, or her2.IgG3. Proliferation was measured by addition of MTS/PMS. Fig. 6 shows the results from a typical assay. mIL-12 and mscIL-12.her2.IgG3 showed an equivalent mitogenic effect on PHA-blasts in a dose-dependent manner. The results are expressed as the mean ± SD of triplicate samples with the background proliferation in medium subtracted. In contrast, her2.IgG3-treated PHA-blasts did not show any proliferation. These results indicate that the mitogenic effect of mscIL-12.her2.IgG3 is due to the IL-12 and not to some other effect by the Ab component of the fusion protein.

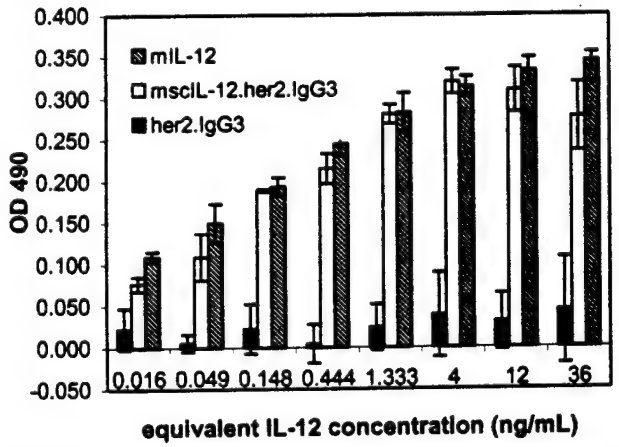

FIGURE 6. Proliferation assays. PHA-activated PBMC were prepared and incubated for 48 h with mIL-12, mscIL-12.her2.IgG3, or her2.IgG3. Proliferation was measured by addition of MTS/PMS and plates were read at OD 490.

Enhanced NK activity

IL-12 has been shown to enhance the cytotoxic action of NK cells. We prepared PBMC (shown by FACS to be 8–9% $CD56^+$, data not shown) and incubated them for 16–18 h with 5 ng/ml mIL-12 or an equivalent IL-12 concentration of mscIL-12.her2.IgG3. The PBMC were also incubated with her2.IgG3 at the same Ab concentration as mscIL-12.her2.IgG3 or medium. These effector cells were added to PKH67-labeled K562 target cells at E:T ratios of 100:1 and 50:1, then incubated for 4 h. After this incubation, PI, which intercalates into the DNA of dead cells, was added and FACS analysis performed. Fig. 7*A* shows a representative FACS result (E:T of 100:1, treated with 5 ng/ml mIL-12) with defined populations of live effectors (*lower left*), dead effectors (*upper left*), live targets (*lower right*), and dead targets (*upper right*) separated into the four quadrants. The *x*-axis measures PKH67 fluorescence intensity and the *y*-axis measures PI fluorescence intensity. A gate was drawn around target cells (PKH67 positive). Figs. 7, *B–E* show histograms of PI fluorescence intensity among target cells gated as in Fig. 7*A*. More positively staining cells (*right*) are dead target cells; less positive cells (*left*) are live target cells. The percent cytotoxicity was calculated from the histograms. The background cytotoxicity in medium was subtracted to give the percent enhanced cytotoxicity. Fig. 7*E* shows that mIL-12 and mscIL-12.her2.IgG3 at equivalent IL-12 concentrations of 5 ng/ml and E:T of both 50:1 and 100:1 comparably enhanced NK cytotoxicity by ~20%, while her2.IgG3 showed no enhancement. These results indicate that the enhanced cytotoxicity by mscIL-12.her2.IgG3 is due to the IL-12 component of the fusion protein and not to some other effect by the Ab component of the fusion protein and that the IL-12 in the fusion protein has activity comparable to rIL-12.

In vivo antitumor activity

After demonstrating that mscIL-12.her2.IgG3 had in vitro biologic activity comparable to rIL-12, the in vivo antitumor activity was investigated using a CT26/Her2 animal model developed in our laboratory (33).

On day 0, CT26/Her2 cells were injected s.c. into the right flank of BALB/c mice. One group of mice was treated with mscIL-12.her2.IgG3, her2.IgG3, or PBS injected i.v. for 5 days beginning

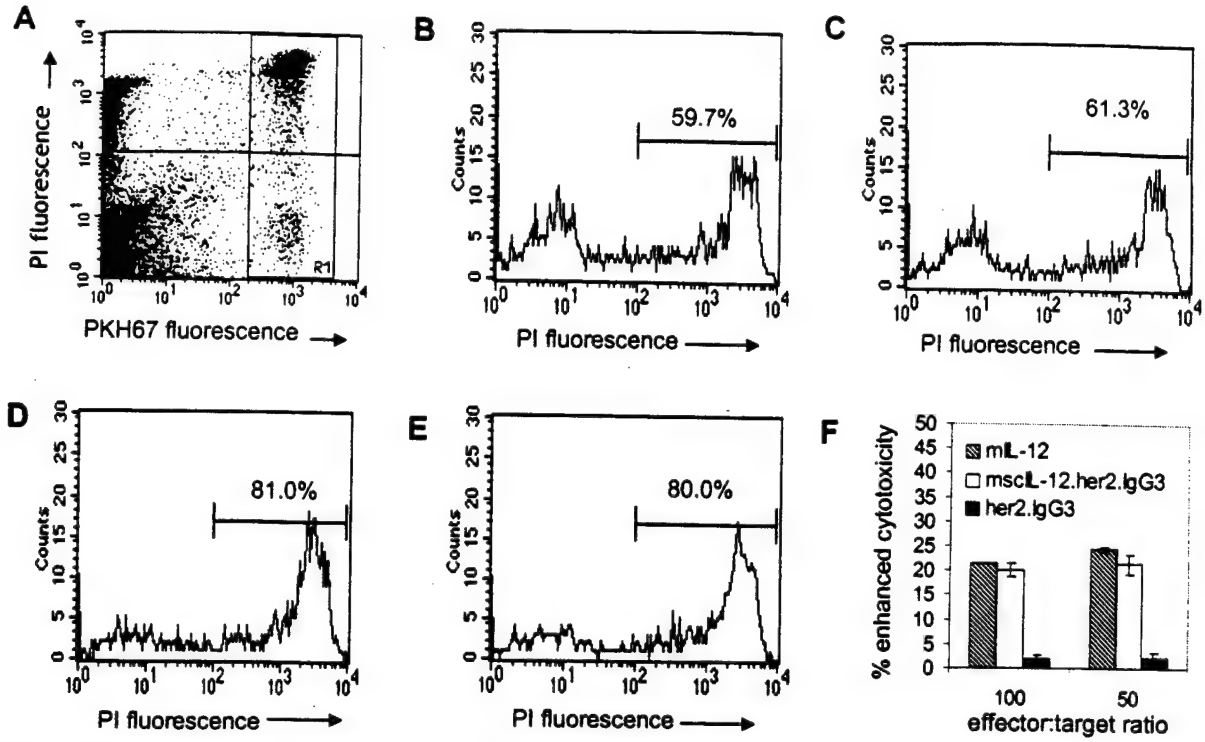

FIGURE 7. NK cytotoxicity assay. *A*, A representative FACS profile (E:T of 100:1, treated with mIL-12) with defined populations of live effectors (LL), dead effectors (UL), live targets (LR), and dead targets (UR). The *x*-axis measures PKH67 fluorescence intensity and the *y*-axis measures PI fluorescence intensity. A gate (R1) is drawn around target cells. *B–E*, Histograms of PI fluorescence intensity among target cells as gated in *A*. More positively staining cells (*right*) are dead target cells; less positive cells (*left*) are live target cells. Effector cells were (*B*) untreated, (*C*) her2.IgG3 treated, (*D*) mIL-12 treated, and (*E*) mscIL-12.her2.IgG3 treated. *F*, The percent live and dead target cells was determined from the histograms as shown in *B–E*. The percent cytotoxicity was calculated as the number of dead target cells/total number of target cells × 100. The percent enhanced cytotoxicity was calculated as percent cytotoxicity (treated effector cells, as in *C–E*) − percent cytotoxicity (effector cells in media, as in *B*). Results are reported as the mean ± SD of triplicate samples.

on day 1, while a second group was similarly treated beginning on day 6 when the tumors averaged 8–9 mm in diameter. Thus, the studies were designed to examine the effect of mscIL-12.her2.IgG3 on both tumor growth and tumor regression. Treatment with mscIL-12.her2.IgG3 slowed the growth of tumors when it began on day 1 (Fig. 8*A*) and arrested tumor growth when it began on day 6 (Fig. 8*C*) compared with mice treated with PBS or her2.IgG3. The tumor weights were used as a more objective indicator of tumor size and confirm the results of the caliper measurements (Fig. 8, *B* and *D*).

These results demonstrate that mscIL-12.her2.IgG3 has significant antitumor activity in immunocompetent mice. Further studies are in progress to determine whether this effect can be seen in other tumor models and to determine the mechanism of the observed antitumor activity.

Discussion

In these studies, we describe the construction and expression of a novel bioactive mscIL-12 IgG3 Ab fusion protein. In the design of our Ab-IL-12 fusion protein, a number of factors were considered. Although our long-term goal is the production of Ab fusion proteins for therapeutic use in humans, mIL-12 was used for these initial studies because it has activity on both human and murine cells, while hIL-12 has activity only on human cells. The use of mIL-12 makes it possible not only to carry out assays using human PBMC to test biologic activity, but also to perform in vivo studies using immunocompetent mice to examine the effects against Her2/*neu*-expressing murine tumors.

Previous studies suggested that an accessible N terminus of the p40 subunit is important for IL-12 bioactivity. When Lieschke et al. constructed a scIL-12, the order of the subunits was found to affect the IL-12 biologic activity (45). When the p35 subunit came before the p40 subunit, there was greatly decreased IL-12 activity; in contrast, when the subunits were reversed, with p40 in front of p35, the scIL-12 had biologic activity comparable to rIL-12 (45–47). Similarly, in an OVA-IL-12 fusion protein in which the p40 subunit was fused to OVA, a 50-fold lower IL-12 activity was observed (48). Constraint of the p40 subunit in a fusion protein may disrupt the interaction between IL-12 and the IL-12R. The IL-12R complex consists of two chains, $\beta 1$ and $\beta 2$, with $\beta 1$ necessary for hIL-12 signaling and activity (49). It is thought that IL-12 interacts with the hIL-12R $\beta 1$ primarily through domains on the p40 subunit (50).

Given the need for an accessible p40 subunit, we chose to fuse the scIL-12 to the amino terminus of the H chain. We were concerned that if we fused the mIL-12.p40.linker.Δp35 to the carboxyl terminus of the Ab H chain, we would constrain the p40 subunit and lose IL-12 activity. In previous studies, it was found that both nerve growth factor (51) and B7.1 (36) had to be joined to the amino terminus of the Ab to maintain their activity in Ab fusion proteins; fusion at the carboxyl terminus of the H chain resulted in impaired activity in both cases. We find the IL-12 in our Ab-IL-12

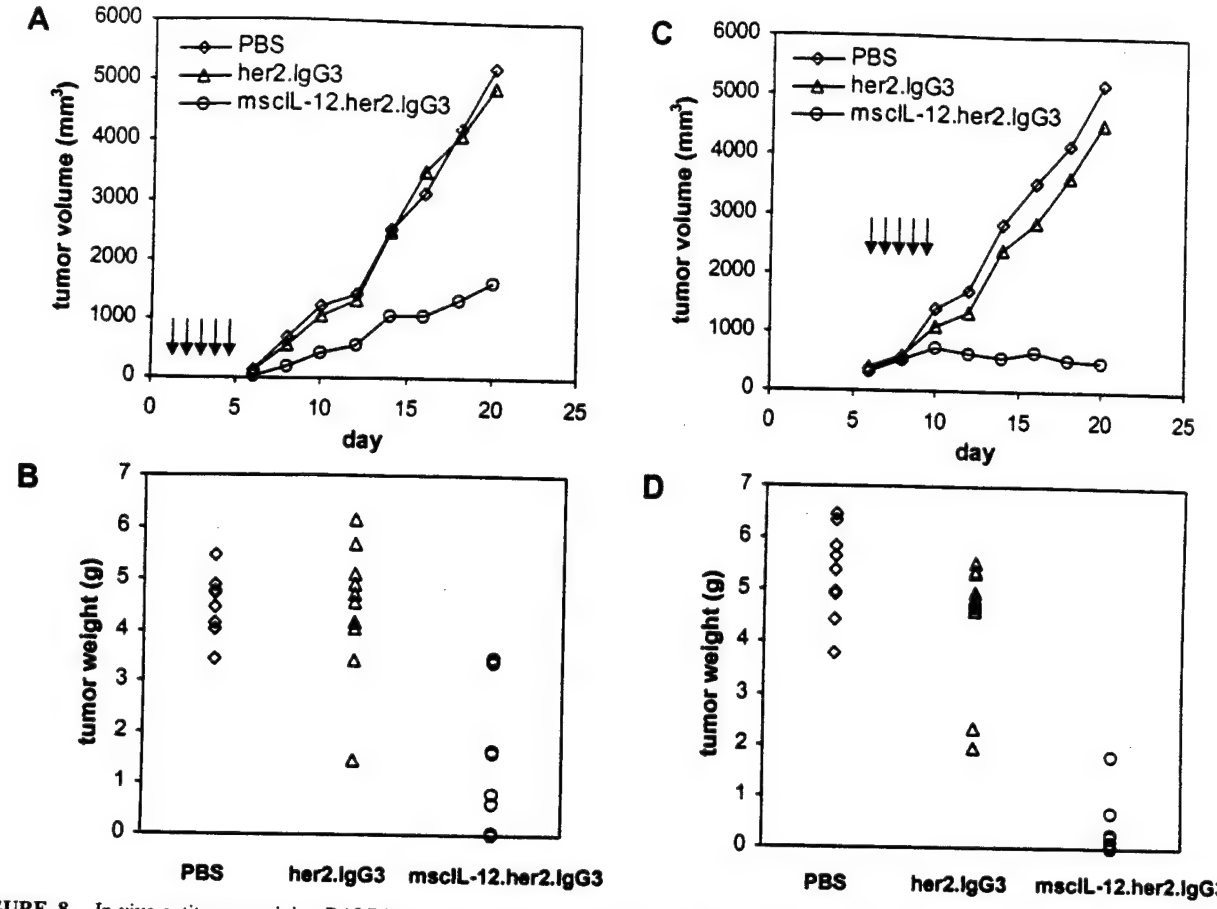

FIGURE 8. In vivo antitumor activity. BALB/c mice were injected with 1×10^6 CT26/Her2 cells s.c. on day 0. Beginning on day 1 or day 6, groups of 10 mice were treated i.v. with either mscIL-12.her2.IgG3 (at a concentration equivalent to 1 μg IL-12/day), her2.IgG3 (at a concentration equivalent to the Ab concentration of mscIL-12.her2.IgG3 administered/day), or PBS for 5 days. Tumor growth was measured with a caliper beginning on day 6, and tumor volume was calculated. The average tumor volumes of the 10 mice used per treatment arm in the groups treated beginning on day 1 and day 6 were plotted against time in *A* and *C*, respectively. At day 20, the mice were sacrificed and the tumors were harvested and weighed. The weights of individual tumors are plotted in *B* and *D* for the groups treated beginning on day 1 and 6, respectively.

fusion protein to be fully functional with IL-12 bioactivity comparable to rIL-12.

Our studies contrast with the work of Gilles et al., who fused the p35 subunit to the carboxyl terminus of the H chain and expressed the p40 subunit from a separate vector (32). While this approach led to the production of functional fusion proteins, the IL-12 had only one-half of the expected bioactivity. In contrast to the single-chain approach, this approach requires the separate transfection of the two IL-12 subunits and does not guarantee that they are present in equimolar concentrations. Although the p40 subunit was not fused to the Ab in Ab-IL-12 fusion protein produced by Gilles et al., the fusion of the p35 subunit to the carboxyl terminus of the Ab without any type of flexible linker may make the p40 subunit somewhat less accessible for receptor binding; this could explain the 2-fold lower IL-12 activity they observed.

Both scIL-12 (75 kDa) and H chain (60 kDa) are large. However, by providing a flexible linker between the two polypeptides, we were able to maintain the activity of both. The presence of IL-12 at the amino terminus of the V_H region does not sterically hinder the ability of the combining site of the Ab to interact with Ag on the cell surface and remain bound (see Figs. 3 and 4). Similarly, the IL-12 in the fusion protein appears to be unaffected in its ability to bind the IL-12R and exhibit IL-12-mediated cellular activation (Figs. 5–7). The attachment of the IL-12 to the V region of the Ab should position it near the surface of the tumor cell and may further potentiate the antigenicity of the targeted tumor.

The ultimate goal of the construction of mscIL-12.her2.IgG3 is its use as an antitumor agent. Using a CT26/Her2 tumor model previously developed in our laboratory (33), our initial in vivo studies demonstrate that this fusion protein has significant antitumor activity in immunocompetent BALB/c mice (Fig. 8). We observed better antitumor activity when treatment was started after the tumors were established with a mean diameter of 8–9 mm than when treatment was started the day after inoculation with tumor cells. This lends support to previous studies by others (4, 52, 53) in which better antitumor activity of IL-12 was observed when tumors were established. They proposed that this may be because effector cells are first recruited to the tumor site and are then activated by IL-12. Further work is being conducted to determine whether the in vivo efficacy we have observed is due to activated T or NK cells, whether a Th1 response has been stimulated, and whether any other antitumor activities may have been stimulated by treatment with mscIL-12.her2.IgG3.

In conclusion, we have demonstrated that it is possible to genetically engineer and express a scIL-12-Ab fusion protein that retains Her2/*neu* Ag specificity and IL-12 biologic activity comparable to rIL-12. Our results indicate that the bulky size of IL-12 does not affect Ag binding and that the Ab does not hinder cytokine receptor binding. Further, this fusion protein demonstrates

antitumor activity in a tumor model using CT26/Her2 cells in syngeneic immunocompetent BALB/c mice. Thus, this Ab-IL-12 fusion protein may be an effective alternate to systemic administration of IL-12 for the treatment of metastatic breast cancer. Using the tumor-targeting ability of the Ab, it should be able to achieve effective local IL-12 concentration at the sites of tumors and metastases with lower doses of IL-12, thus decreasing the risk of toxicity associated with IL-12 treatment. An anti-Her2/*neu* mAb has had success in clinical trials for the treatment of Her2/*neu*-expressing metastatic breast cancer (20). Fusion of a cytokine-like IL-12 that has antitumor and antimetastatic properties to a Her2/*neu*-specific Ab may enhance its efficacy, particularly if it elicits a tumor-specific immune response.

Acknowledgments

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A murine B cell lymphoma expressing human HER2/*neu* undergoes spontaneous tumor regression and elicits anti-tumor immunity[1].

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[3] The abbreviations used are: IL-12, interleukin-12; Id, idiotype; TAA, tumor-associated antigen; IMDM, Iscove's modification of Dulbecco's medium; SCID, severe combined immunodeficiency; MoMuLV, Moloney murine leukemia virus; neo, neomycin-resistance; DNS, N,N dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); ECD^{HER2}, extracellular domain of HER2/*neu* antigen; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate buffered saline; AP, akaline phosphatase; HBSS, Hanks's balanced salt solution; IgG immunoglobulin G; CEA, carcinoembryonic antigen.

[4] Unpublished results.

ABSTRACT

In the present study we describe a novel murine tumor model in which the highly malignant murine B-cell lymphoma 38C13 has been transduced with the cDNA encoding human tumor associated antigen HER2/*neu*. This new cell line (38C13-HER2/*neu*) showed stable surface expression but not secretion of human HER2/*neu*. It also maintained expression of the idiotype (Id) of the surface immunoglobulin of 38C13 which serves as another tumor associated antigen. Surprisingly, spontaneous tumor regression was observed following s.c., but not i.v. injection of 38C13-HER2/*neu* cells in immunocompetent syngeneic mice. Regression was more frequently observed with larger tumor cell challenges and was mediated through immunological mechanisms because it was not observed in syngeneic immunodeficient mice. Mice that showed complete tumor regression were immune to challenge with the parental cell line 38C13 and V1, a variant of 38C13 that does not express the Id. Immunity could be transferred with sera suggesting that an antibody response mediated rejection and immunity. Continuously growing s.c. tumors as well as metastatic tumors obtained after the i.v. injection of 38C13-HER2/*neu* maintained expression of human HER2/*neu* which can serve as a target for active immunotherapy. As spontaneous tumor regression has not been observed in other human murine models expressing human HER2/*neu*, our results illustrate the enormous differences that can exist among different murine tumors expressing the same antigen. The present model provides a useful tool for the study of the mechanisms of protective immunity to B-cell lymphoma and for the evaluation of different therapeutic approaches based on the stimulation or suppression of the immune response.

INTRODUCTION

The HER2/*neu* proto-oncogene (also known as c-erbB-2) encodes a 185 kDa transmembrane glycoprotein receptor that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain (1-3). HER2/*neu* is expressed at low levels on some normal cells, however, markedly increased expression has been observed in many human breast, gastrointestinal, lung and ovarian cancers (4-8). The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent candidate for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA), leads to an objective response in a subset of patients with tumors overexpressing the HER2/*neu* oncoprotein (9-11). These results justify recent enthusiasm for continued efforts to refine existing approaches and to develop new strategies that target HER2/*neu*.

We have developed a family of anti-human HER2/*neu* antibody fusion proteins containing immunostimulatory molecules such as the cytokine IL-12[3] (12), costimulatory molecules such as B7.1 (13) or chemokines such as RANTES (14). To evaluate the immunological efficacy of these proteins, it is critical that tumors expressing the target antigen can grow in immunologically competent mice. To produce murine tumors expressing human HER2/*neu*, we transduced the murine colon adenocarcinoma cell lines CT26 and MC38 and the murine T cell lymphoma EL4 with the cDNA encoding the human HER2/*neu*. We showed that those cells were able to grow in immunocompentent mice while maintaining the expression of human HER2/*neu* (15) and such models are now being used for preclinical evaluation of the efficacy of anti-HER2/*neu* antibody fusion proteins (12), (Penichet *et al,* unpublished results)[4].

To further expand our repertoire of human HER2/*neu* expressing murine tumors, we have developed a new model using the highly malignant murine B-cell lymphoma 38C13. Although overexpression of HER2/*neu* has been mainly associated with breast, gastrointestinal, lung and ovarian cancers (4-8), it has also been described for B cell lymphoma (16, 17). Thus, 38C13 expressing human HER2/*neu* may be used to evaluate the efficacy of antibody fusion proteins against B-cell lymphoma. If these proteins are effective they can be further evaluated in clinical trials targeting lymphomas expressing HER2/*neu* or they can be modified to target other TAA found on B-cell lymphomas. In addition, since the Id of the surface immunoglobulin of 38C13 has been previously used to target anti-Id antibody fusion proteins (18, 19), the 38C13-HER2/*neu* model will allow us to test the potential synergistic effect of different antibody fusion proteins targeting two different TAA: the Id and HER2/*neu.* Moreover, the availability of different cell lines expressing the same antigen allows us to test the efficacy of HER2/*neu* targeted approaches in a variety of cell lines and/or mouse strains and it is well known that different responses to the same anti-cancer therapy are exhibited by different tumors (19).

In this report, we describe the transduction of 38C13 with a retroviral construct containing the full length cDNA encoding the human *HER2/neu* gene. These cells (38C13-HER2/*neu*) show stable expression of human HER2/*neu* on their surface while maintaining expression of the surface immunoglobulin. The biological properties of this transduced cell line were analyzed after transplantation into immunologically intact syngeneic mice. Parameters that were investigated include tumor growth rate and phenotype, ability to produce metastases, expression of HER2/*neu*, antigen shedding and the anti-human HER2/*neu* response of the host. Contrary to our expectations, spontaneous tumor regression after temporary growth was observed followed s.c. injection of tumor cells and this regression was more frequently observed the greater the number of cells used to elicit tumor growth; however, regression was not observed following i.v. injection of tumor cells.

MATERIALS AND METHODS

Cells: 38C13 is a C3H/HeN murine B cell lymphoma expressing a surface μκ antibody (Id) that arose in a carcinogen (7,12-dimethylbenz(a)anthracene) treated mouse (20, 21). V1 is an Id-negative variant derived from the original 38C13 tumor (22). Both cell lines were kindly provided by Drs. Ronald and Shoshana Levy (Stanford University, Stanford, CA). The parental and transduced cells were maintained in IMDM (Irvine Scientific Inc, Irvine, CA) supplemented with 10 % iron supplemented calf serum (Atlanta Biologicals, Norcross GA) at 37°C with 5% CO_2.

Mice: Female immunocompetent C3H/HeN mice and Rag2 double knockout mice lacking mature T and B cell lymphocytes with the C3H/HeN background between 6-8 weeks of age obtained from Taconic Farms Inc (Germantown, NY) were used. The mice received food and water ad libitum. Artificial light was provided under a 12/12 h light/dark cycle. The temperature of the facility was 20°C with 10-15 air exchanges per hour. All experiments were performed according to National Institutes of Health (NIH) (Bethesda, MD) *Guide for the Care and Use of Laboratory Animals.*

Retroviral expression vector, transduction and screening: The cDNA for HER2/*neu* cloned in a MoMuLV-based retroviral vector including the *neo* gene under the control of the SV40 promoter was used for transduction of 38C13. This vector was previously used to derive other human HER2/*neu* expressing murine cell lines (15). Cells were selected with geneticin (Sigma Chemical, St. Louis, MO). HER2/*neu* expression on the surface of transduced cells was detected with an immunofluorescence assay. 10^6 cells were

incubated with 1 μg of a recombinant anti-HER2/*neu* antibody for 2 h at 4°C. Recombinant anti-Id and recombinant anti-DNS antibodies were used as positive and negative isotype matched controls respectively. All three recombinant antibodies were developed in our laboratory and contained human κ and γ3 constant regions. Cells were washed and following incubation for 2h at 4°C with biotinylated goat anti-human IgG (Pharmingen, San Diego, CA), then were washed and incubated for 30 min with PE-labeled streptavidin (Pharmingen, San Diego, CA). Analysis was performed by flow cytometry with a FACScan (Becton-Dickinson, Mountain View, CA) equipped with a blue laser excitation of 15 mW at 488 nm.

Determination of the presence of ECD^{HER2} in cell culture supernatant: Circulating ECD^{HER2} was detected using an ELISA. 96-well microtiter plates were coated with 50 μl of recombinant human anti-HER2/*neu* IgG3 (developed in our laboratory) at a concentration of 1 μg/ml. The plates were blocked with 3% BSA in PBS and dilutions of cell culture supernatant or ECD^{HER2} (kindly provided by Dr. James D. Marks, UCSF; San Francisco, CA) in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. The wells were then washed with PBS and incubated for 2 h at room temperature with the anti-HER2/*neu* MAb Neu 9G6 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). After washing with PBS, alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) was added and the plates were incubated for 1h at 37 °C. After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma Chemical, St. Louis, MO) was added to the wells for 1 h and plates were read at 410 nm.

s.c. and i.v. transplantation of tumor cell lines: Mice were injected s.c. in the right flank with 38C13-HER2/*neu* in 0.15 ml of HBSS (Gibco BRL, Grand Island, NY). In

order to compare the s.c. growth of the transduced cell lines with their respective parental cell line, additional groups were injected with the same dose of 38C13. Tumor growth was measured three times a week with a caliper and length of survival recorded. Mice were injected i.v. with 38C13-HER2/*neu* or with the corresponding dose of 38C13 in 0.3 ml of HBSS (Gibco BRL, Grand Island, NY) via the lateral tail vein and length of survival recorded.

Histologic study: Mice were injected s.c. with 38C13-HER2/*neu* or 38C13 and tumor growth monitored described above. 24 h after tumor regression started, mice were euthanized and histologic study of their respective tumors was carried out on paraformaldehyde-fixed paraffin-embedded tissue samples. Sections of 6 μm were stained with hematoxylin/eosin.

Passive transfer of sera and splenocytes: A pool of sera and splenocytes was prepared from mice which had shown tumor regression and survived 60 days without new evidence of tumor following injection with 38C13-HER2/*neu*. According to previous reports, mice inoculated with 38C13 that are free of tumor, 60 days after the injection are, in fact, cured of lymphoma (19, 23, 24). A splenocyte suspension was prepared by mincing and compressing freshly resected spleens between two slides on a petri dish in the presence of HBSS. The splenic capsule was discarded and the cell suspension was transferred to a polystyrene tube. After washing twice with HBSS, the cells were counted using crystal violet staining (Sigma Chemical, St. Louis, MO). To transfer immunity using splenocytes, 5×10^7 cells in 0.3 ml of HBSS were injected into the tail vein of naïve syngeneic recipient mice. All mice receiving splenocytes were injected i.p. with 100 IU of Heparin (Sigma Chemical, St. Louis, MO) 30 min before the transfer of the splenocytes. To transfer immunity using sera, 0.3 ml of pooled sera was injected into the

tail vein of naïve syngeneic recipient mice. One day later, mice were challenged s.c. with a lethal dose (10^4) of the parental tumor 38C13 cells. Tumor incidence and survival were monitored. Naïve syngeneic recipient mice that did not receive splenocytes or sera or that received splenocytes or sera from naïve mice of a similar age to the long-term survivors were used as controls.

Determination of murine anti-HER2/*neu* and anti-Id antibodies: The presence of antibodies to human HER2/*neu* or to murine Id in mice sera was determined by ELISA using 96-well microtiter plates coated with 50 μl (at a concentration of 1 μg/ml) of ECD^{HER2} or with Id obtained from concentrated supernatant of the hybridoma A1-2 which secretes high levels of soluble 38C13 Id (25). The plates were blocked with 3% BSA in PBS and dilutions of serum in PBS containing 1% BSA were added to the wells and incubated overnight at 4°C. The wells were then washed with PBS, alkaline-phosphatase goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) added and the plates were processed as described above. As a negative control for determining anti-HER2/*neu* titers, we used sera from mice of the same age bearing tumors of non-transduced 38C13. As a negative control for determining anti-Id titers we used sera from naïve mice of the same age. All ELISAs for comparison of titers between 38C13 and 38C13-HER2/*neu* were made simultaneously in duplicate and using an internal positive control curve for each plate.

Detection of HER2/*neu* and Id expression in tumors by flow cytometry: Single cell suspensions from 38C13-HER2/*neu* and 38C13 were prepared by mincing and pipetting freshly isolated tumors in cold medium. The detection of Id and HER2/*neu* surface expression on a fresh single cell supension, as well as on the same cells kept for one week in tissue culture was done by flow cytometry as described above.

Statistical analysis: Statistical analysis of the differential findings between experimental groups of mice was done using the nonparametric Wilcoxon-Mann-Whitney rank sum test. This allows us to include both dead mice as well as the long-term survivors in the analysis.

RESULTS

***In vitro* human HER2/*neu* expression in transduced cells:** The murine tumor cell line 38C13 was transduced with the retroviral vector containing the HER2/*neu* cDNA under the control of the MoMuLV enhancer/promoter (38C13-HER2/*neu*). A stable pool of cells selected in geneticin was tested for surface expression of human HER2/*neu* by flow cytometry. Human HER2/*neu* expression was detected on the surface of the transduced cell line (Fig. 1, panel B). Incubation of the anti-HER2/*neu* IgG3 with 38C13-HER2/*neu* in the presence of excess soluble ECD^{HER2} abrogated binding (data not shown) confirming the specificity of the recombinant anti-HER2/*neu* IgG3 used in this assay. The expression of the Id of the μκ surface immunoglobulin was also detected by flow cytometry (Fig. 1, panel C). The level of Id expression in 38C13-HER2/*neu* was similar to the level found on the parental cell line (Fig. 1, panel F).

The above results confirmed the surface expression of human HER2/*neu*. Secretion of the ECD^{HER2} has been reported for some HER2/*neu* expressing tumors (26-28). To address this issue, we quantified the amount of ECD^{HER2} present in the culture supernatant of 38C13-HER2/*neu* cells grown at 10^6/ml and incubated for 24 h. Although the ELISA assay used can detect more than 2 ng/ml of soluble recombinant ECD^{HER2}, we did not detect the presence of the ECD^{HER2} in culture supernatants of cells carried in tissue culture or isolated from tumors and expanded *in vitro* (data not shown).

s.c. tumor growth characteristics: The growth kinetics in the s.c. space of normal syngeneic mice of 38C13-HER2/*neu* was compared to that of the parental cell line 38C13. Doses of 10^3 and 10^4 38C13 cells injected s.c. have been shown to yield tumors in 100% of mice (18, 19, 23-25, 29). For this reason, we injected groups of 5 mice in the right flank with 10^3,10^4, 10^5 or 10^6 38C13-HER2/*neu* or 38C13 cells. 100% of the mice developed tumors with a similar time of tumor onset for each dose of 38C13-HER2/*neu* and 38C13 (Fig. 2). As expected, higher doses of both cell lines resulted in shorter latency. However, when the tumors reached a size of approximately 1 cm in diameter, some of the 38C13-HER2/*neu* tumors showed spontaneous regression. This phenomenon appeared to be dose-related as complete regression was more frequently observed with larger tumor cell challenges: 1/5 for 10^3, (20%), 4/5 for 10^4 (80%), 4/5 for 10^5 (80%) and 5/5 for 10^6 (100%) (Fig. 3 and Table 1). All mice showing complete tumor regression became long-term survivors. As expected all mice injected with 10^3, 10^4 or 10^5 38C13 cells developed progressive tumors and died (Figs 2 and 3). Surprisingly, one of the five mice injected with 10^6 38C13 cells also showed spontaneous tumor rejection and became a long-term survivor. The survival of mice inoculated with 10^4, 10^5 and 10^6 38C13-HER2/*neu* cells was significantly better than that of mice injected with 38C13 ($P < 0.05$). Although the survival of mice injected with 10^3 cells was not significantly different between 38C13-HER2/*neu* and 38C13, one of five mice injected with 38C13-HER2/*neu* became a long-term survivor, while all of the mice injected with 38C13 cells died. Postmortem studies of mice injected with s.c. 38C13 or 38C13-HER2/*neu* tumors revealed the presence of metastatic tumors in lymph nodes throughout the body (data not shown).

A repetition of this experiment using 10 mice per group for injections of 10^3, 10^4 or 10^5 cells and 20 mice per group for injection of 10^6 cells gave similar results (Table 1). Larger tumor cell challenges of 38C13-HER2/*neu* were associated with more frequent complete spontaneous tumor regression. As expected, the injection of 10^3, 10^4 or 10^5

38C13 cells resulted in progressively growing tumors in 100% of mice, but following the injection of 10^6 38C13, spontaneous tumor regression was seen in 2 of 20 (10%) mice.

Two months after the s.c. tumors regressed long-term survivors were challenged with the 10^4 38C13 cells in the left flank. At this tumor cell dose all naïve mice of the same age showed progressive tumor growth and died. In contrast, 100% of the long-term survivors previously injected with 10^4, 10^5 or 10^6 38C13-HER2/*neu* remained free of tumor (Table 2). Similar results were observed with 2 of the 3 (67%) mice previously injected with 10^3 38C13-HER2/*neu* suggesting that this lower dose is not only associated with less frequent spontaneous tumor regression but also with weaker anti-tumor immunity. When long-term survivors previously injected with 10^6 38C13-HER2/*neu* were challenged with a lethal dose of 10^4 V1 cells, an Id-negative variant derived from the original 38C13, 100% of mice remained free of tumor while all naïve mice of the same age showed progressive tumor growth and died (Table 2).

To compare the histology of regressing and non-regressing tumors, groups of 5 mice were injected s.c. in the right flank with 10^6 38C13-HER2/*neu* or 38C13. As expected, all of the mice initially developed tumors (data not shown). By day 11 after the injection of the cells, regression had begun in all of the mice injected with 10^6 38C13-HER2/*neu*. No tumor regression was observed in mice injected with 10^6 38C13. Figure 4 shows histologic sections stained with hematoxylin/eosin of tumors obtained from mice 12 days following injection. The 38C13 tumor is a highly cellular tumor that infiltrates the subcutaneous space and part of the muscular coat (panel A). Examination of regressing 38C13-HER2/*neu* tumor was characterized by intense eosinophilia, cell shrinkage, loss of structure and fragmentation, all classic images of necrosis (30) (panel B). Similar results were observed in all mice injected with 38C13-HER2/*neu* cells (data not shown). All mice injected with 38C13 had highly cellular tumors similar to the tumor depicted in Fig. 4, panel A.

To test whether tumor regression was immunologically mediated, groups of 8 syngeneic Rag2 double knockout mice which lack mature T and B lymphocytes received s.c. injections of 10^6 38C13 or 38C13-HER2/*neu* in the right flank. In contrast to what had been oberved using immunocompetent mice, both cell lines showed similar tumor growth and no tumor regression was observed in mice injected with 38C13 or 38C13-HER2/*neu* (data not shown).

Passive transfer of sera and splenocytes from long-term survivors: To determine if humoral or cellular immunity was responsible for the protection observed in mice showing spontaneous tumor regression, either serum or splenocytes from mice that had previously rejected s.c. 38C13-HER2/*neu* tumors was transferred to naïve C3H/HeN mice 24 h prior to tumor challenge. Transfer of splenocytes or serum was found to confer significant protection to challenge 24 h later with 10^4 38C13 cells causing either retardation or resistance to tumor growth (Fig 5). Survival of mice receiving splenocytes or serum from immune mice was significally greater than the control group that did not receive treatment: $P < 0.001$ for mice receiving splenocytes and $P = 0.01$ for mice receiving serum. Mice receiving a similar amount of serum or splenocytes from naïve mice showed survival curves similar to mice that did not receive any treatment (data not show).

i.v. tumor growth characteristics: The *in vivo* studies described above have been restricted to tumors growing in the s.c. space. However, as the route of injection can influence the growth potential of certain cell lines, we also investigated the growth of 38C13-HER2/*neu* after i.v. injection. Groups of 5 mice were injected i.v. with 10^3,10^4, 10^5, or 10^6 38C13-HER2/*neu* or 38C13 cells. We found that after injecting the cells i.v., all of the mice injected with 38C13-HER2/*neu* or 38C13 developed a disseminated

malignant disease leading to death (Fig. 6). No long-term survivors were observed. Postmortem studies revealed the presence of tumor metastases in the lymph nodes throughout the body in all mice. Similar results were obtained when this experiment was repeated (data not show).

Determination of the anti-human HER2/*neu* and anti-Id response in mice bearing s.c. and i.v. tumors: To determine if anti-human HER2/*neu* or anti-Id antibodies were elicited in mice bearing s.c. tumors, groups of 5 mice inoculated s.c. with 10^4 38C13-HER2/*neu* or 38C13 tumor cells were bled every 3 days for 15 days following injection of the cells. Table 3 shows that anti-human HER2/*neu* antibodies were seen in all of the mice bearing HER2/*neu* expressing tumors by day 12 with response detectable in 2/5 (40%) of the mice 6 days after challenge. The sera were also tested by ELISA for the presence of anti-Id (Table 3). We did not detect anti-Id in mice bearing the parental tumor 38C13. However, in mice bearing 38C13-HER2/*neu* tumors a detectable anti-Id response was observed in 3/5 (60%) by day 12. This anti-Id immune response was observed in 3 of the 4 mice showing tumor regression but not in the mouse with a continuously growing 38C13-HER2/*neu* tumor.

To compare the titers of anti-human HER2/*neu* or anti-Id antibodies elicited in mice bearing s.c. tumors induced by different doses of cells, groups of 5 mice (randomly selected) injected in the right flank with 10^3, 10^4,10^5 or 10^6 38C13-HER2/*neu* or 38C13 cells were bled 12 days after the injection of the cells and the sera analyzed for the presence of anti-human HER2/*neu* or anti-Id antibodies (Table 4). No anti-human HER2/*neu* or anti-Id was detected in sera collected from mice injected with 10^3 38C13-HER2/*neu* . However, at higher doses of 38C13-HER2/*neu* cells, both anti-human HER2/*neu* and anti-Id antibodies were seen. Consistent with the results presented in Table 3, mice injected with 10^4 38C13 do not show an anti-Id response day 12 while 3/5

of mice injected with 10^4 38C13 showed anti-Id titers. Inoculation with higher doses (10^5 or 10^6) of 38C13-HER2/*neu* results in higher anti-Id titers compared with equivalent dose of 38C13. It is important to note that no direct correlation is seen between the magnitude of the anti-human HER2/*neu* response and the survival of mice, suggesting that anti-human HER2/*neu* antibody response may not significantly contribute to survival. In contrast, the anti-Id response may be associated with an effective anti-tumor response. For mice injected with 10^6 38C13-HER2/*neu* cells, the only mouse showing tumor progression exhibited the lowest anti-Id titer. In the group of mice injected with 10^5 38C13-HER2/*neu* cells, the only mouse showing tumor regression exhibited the highest anti-Id titer. In the group of mice injected with 10^4 cells, one of the three mice showing tumor regression exhibits the highest anti-Id titer while the other two mice exibit titers similar (0 or 50) to mice showing tumor progression. No anti-Id was detected in mice injected with 10^3 38C13-HER2/*neu* even though two mice showed tumor regression. However, we should stress that at day 12 tumor regression had not yet begun in mice injected with 10^3 or 10^4 cells, while mice injected with 10^5 or 10^6 cells already showed clear signs of regression or progression. Thus, the day 12 response may not accurately reflect the association between anti-Id titers and the anti-tumor response.

Table 4 also shows that no anti-Id was seen in sera collected from day 12 mice injected with 10^3 or 10^4 parental cells 38C13. However, increasing the injection dose of 38C13 cells resulted in detectable titers of anti-Id antibodies in 3/5 (60%) mice challenged with 10^5 38C13 and in 5/5 (100%) of mice challenged with 10^6 38C13. These anti-mouse Id titers however, are clearly lower that than those found in mice challenged with 10^4, 10^5, 10^6 38C13-HER2/*neu* and were not associated with tumor regression.

We also studied the anti-human HER2/*neu* and anti-Id response elicited by day 12 in mice injected with 10^3, 10^4,10^5 or 10^6 38C13-HER2/*neu* i.v. (Table 5). Interestingly, anti-human HER2/*neu* titers are present in 4/5 mice injected with 10^3 38C13-HER2/*neu,* however, in contrast with what were seen in mice injected s.c., increasing the injection

dose did not increase the anti-human HER2/*neu* titers. No anti-Id response was detected in any mice injected with 10^3, 10^4 or 10^5 38C13-HER2/*neu* cells and only a modest response was observed in 3/5 (60%) of mice injected with 10^6 38C13-HER2/*neu* cells.

***In vivo* tumor expression of HER2/*neu* and Id as detected by flow cytometry:** Flow cytometry analysis of freshly isolated cells from 15 day old s.c. (Figure 7, panel A and B) or metastatic (Fig. 7, Panels C and D) 38C13-HER2/*neu* tumors growing in cervical lymph nodes from four different mice inoculated with 10^4 cells showed persistence of cell surface expression of human HER2/*neu* in all tumors. However, the level of surface expression of human HER2/*neu* determined immediately following removal of tumor from the mice appears decreased compared with cells maintained in tissue culture. After one week in culture the level of human HER2/*neu* detected markedly increased (Fig. 7, panels E-H) although in some cases not to the same level seen with cells maintained continuously in tissue culture. The level of surface expression of Id from the freshly isolated tumors described above appears to be identical to that of cells maintained in tissue culture (data not shown). Similar results have been observed with cells isolated from 8 additional continuously growing s.c. 38C13-HER2/*neu* tumors dissected from different mice injected s.c. and from 8 additional metastatic tumors growing in the lymph nodes of mice injected i.v. (data not shown). We have also confirmed that the level of HER2/*neu* and Id expression is similar among 3 different metastatic tumors growing in the same mouse and from 4 tumors from different mice injected i.v. (data not shown). The above results suggest that no *in vivo* selection of variants lacking the expression of human HER2/*neu* or Id does not occur.

DISCUSSION

The 38C13 B-cell lymphoma was successfully transduced with a retroviral construct containing the full length cDNA encoding human HER2/*neu*. We found that the transduced cells show stable high level surface expression of both TAAs: human HER2/*neu* and Id. We also found no secretion of soluble HER2/*neu*. ECD^{HER2} is known to be released by some cancer cells that overexpress HER2/*neu* (26-28) and elevated ECD^{HER2} serum levels have been described in patients with breast cancer (26, 31). The secretion of ECD^{HER2} has been reported to be a drawback for anti-HER2/*neu* therapy in humans. However, the dose of the rhuMAb HER2 now in clinical use provides adequate serum concentrations in all patients except those with serum levels of tumor-shed $ECD^{HER2} \geq 500$ ng/ml (9, 10). Tumor-shed Id has been described as a significant limitation for antibody based therapeutic approaches targeting the Id expressed on lymphomas (25). Mice bearing the 38C13 tumors accumulate only a small amount of antibodies bearing the Id during the first week following injection of a relatively small number of cells, but after the tumor becomes established the level of Id protein detected in serum increases at a logarithmic rate making the treatment of established tumors very difficult (25). The lack of tumor-shed ECD^{HER2} by our HER2/*neu* expressing cell lines indicates that secretion of this TAA will not interfere with assessment of antibody or antibody fusion protein treatments targeting HER2/*neu*.

In previous studies, we found that expression of human HER2/*neu* on the surface of murine CT26 and MC38 adenocarcinomas and EL4 T-cell lymphoma does not significantly change the *in vivo* growth properties or morphology of these cells (19). These *in vivo* properties dramatically contrast with the cell death and complete tumor regression resulting in permanent immunity to further challenge observed in s.c. 38C13-HER2/*neu* tumors. This regression was effected through immunological mechanisms since it was not observed in syngeneic immunodeficient mice. These differences in

growth characteristics appear to be a consequence of either the nature of the parental cells and/or the mouse strain rather than the level of HER2/*neu* expression which is similar in all tumor models (data not shown). The same HER2/*neu* expression vector, MoMuLV-based retroviral vector including the *neo* gene under the control of the SV40 promoter was used to produce all the HER2/*neu* expressing cell lines.

The ability to transfer immunity into naïve mice using serum from immune mice suggests that an antibody mediated mechanism is at least partially responsible for tumor rejection and the maintenance of immunity. This is consistent with other reports that vaccinations using either the Id protein or DNA encoding for Id, induced tumor-protection that can be largely attributed to humoral rather than cellular immunity (32, 33). We also showed that immunity can be transferred by splenocytes from immune mice, however, this is also consistent with the humoral mechanism because 40 % of splenocytes are B cells (34). However, as 35% of splenocytes are T cells (34) we can not exclude the possibility that a T cell immune response might also provide anti-tumor immunity. We have found that transduction of this tumor and other murine tumor models with human HER2/*neu* does not decrease the level of expression of MHC class I, (Penichet et al, unpublished results)[4], suggesting that those cells may be able to elicit targeted cytotoxic T cell response. Further studies are required to define the role (if any) of cellular immunity in tumor rejection and immunity.

Having shown that immunity can be transferred by sera, we characterized the anti-human HER2/*neu* and anti-Id responses. We detected an anti-human HER2/*neu* antibody response in mice bearing HER2/*neu* expressing tumors (s.c. and i.v.). A similar humoral response has also been described in mice bearing primary s.c. or metastatic CT26-HER2/*neu*, MC38-HER2/*neu,* and EL4-HER2/*neu* tumors, although in these models tumor regression was not observed (15). Although the anti-human HER2/*neu* antibodies elicited against the 38C13-HER2/*neu* tumor s.c. tumors may play a role in tumor rejection, we have found no correlation between the level of anti-HER2/*neu* titers

and the fate of the tumor (regression or progression). We also found that continuously growing tumors maintain human HER2/*neu* expression, suggesting that variants lacking the expression of human HER2/*neu* are not selected. An alternative possibility is that the presence of foreign antigens such as HER2/*neu* on the surface of 38C13 serves as an adjuvant to facilitate a humoral immune response against other antigens such as the Id. Anti-Id therapy has been successful in the 38C13 model (18, 19, 23, 24). In fact we have found higher titers of anti-Id antibodies in mice bearing s.c. 38C13-HER2/*neu* compared to mice bearing s.c. 38C13 tumors. Furthermore higher anti-Id titers appear to correlate with tumor regression. However anti-Id antibodies were not detected in some of the mice showing regressing s.c. 38C13-HER2/*neu* tumors. It is possible that in these mice Id shedding by the 38C13 tumors (25) leads to immune complexes decreasing the concentration of anti-Id antibodies in blood and resulting in underestimation of the anti-Id response.

Although the ability to elicit an anti-Id immune response might explain the spontaneous tumor regression, we can not exclude the possibility that other antibodies with unknown specificity have been elicited and that these are partially or totally responsible for tumor regression and immunity. In favor of this hypothesis is the observation that the rejection of 38C13-HER2/*neu* results in immunity to further challenge not only with the parental cell line 38C13, but also with V1, a cell line which is negative for Id expression (22) and insensitive to treatment with anti-Id antibodies (33). This observation suggests that anti-tumor immunity generated after regression of 38C13-HER2/*neu* is directed against one or more common antigens shared by 38C13-HER2/*neu,* 38C13 and V1. Such antigens may be known receptor molecules such as CD19 and CD40 which have been successfully used as targets of antibody based therapy in B cell lymphoma (35) or may be unknown antigens. If unknown antigens are the targets, ,hybridomas from splenocytes of immune mice may provide novel and effective antibodies for the therapy of B-cell lymphoma. Several mechanisms have been described

to explain the anti-tumor activity of anti-B cell lymphoma antibodies such as anti-Id, anti-CD19 or anti-CD40, including antibody dependent cell-mediated cytotoxicity (ADCC) (18, 23, 24) as well as antibody-mediated inhibition of tumor growth (35). Further studies are required to define the mechanism of action of the anti-tumor activity present in the sera of mice that have rejected the 38C13-HER2/*neu* tumor.

Xenogenization is a term used to describe attempts to make tumor cells antigenically foreign to their host (36, 37) and includes the expression of foreign antigens such as viral antigens on the surface of tumor cells to potentiate the host immune reaction against the tumor. The rat fibrosarcoma KMT-17 infected with nonlytic murine leukemia virus (Friend virus), like 38C13-HER2/*neu*, regresses spontaneously in the syngeneic host after an initial period of growth and induces protective immunity to noninfected homologous tumor cells (38). The mechanism of rejection is not fully understood, however, it was found that surface expression of CE7, a non-viral TAA is strongly enhanced following viral infection. This enhanced expression may stimulate a strong anti-tumor response, resulting in acquisition of resistance to parental KMT-17 (38). Although we did not find that alteration in the expression of the Id in the transductant, we cannot rule out the possibilty that there is enhanced expression of other unknown 38C13 TAA antigens.

In addition to expressing HER2/*neu* 38C13-HER2/*neu* cells also express the product of the *neo* gene, the neomycin phosphotransferase, and this phosphotransferase activity can induce changes in the cells (39) which might result in higher immunogenicity. In fact, the transduction of 9L rat glioma with a *neo* gene was associated with a decreased *in vivo* tumor growth in immunocompetent animals, although the mechanism responsible was not defined (40). Although neomycin phosphotransferase is expressed in the intracellular compartment, it is a bacterial gene product and thus potentially can serve as an immunogen. Even though the *neo* gene has been used in vast numbers of *in vivo* experiment without eliciting an immune reaction, one patient

receiving multiple infusions of gene-modified T lymphocytes was shown to develop anti-neo and anti-herpes thymidine kinase cell-mediated immune responses that coincided with rapid disappearance of transduced cells *in vivo* (41). Human HER2/*neu,* the other xenoantigen that we have expressed, shows more than 90% homology with the rodent HER2/*neu* (42). Despite this high degree of homology, its expression appears to be sufficient to cause elicit an anti-human humoral immune response and may trigger the anti-tumor activity. Thus, the anti-tumor immune reaction may be elicited by increased expression of TAAs and/or by the expression of the xenoantigens human HER2/*neu* and/or the *neo* gene product, indeed, it is possible that the xenoantigens act as adjuvants to potentiate the immune response to other TAAs.

Our data suggest that the parental 38C13 cells can be immunogenic and that a s.c. innoculation of a very high dose of 38C13 such as 10^6 cells is able to trigger a protective immune response in a subset (10-20%) of mice. This contrasts with previous observations that s.c. inoculation of increasing numbers of 38C13 tumor cells proportionally shortens the mean survival of injected animals. However, we should stress that these observations were made using doses from 10^3 to 10^4 38C13-tumor cells (18, 23, 24) and we have also found these lower doses to yield tumors in all animals. Apparently a threshold level of antigen must be exceeded in order to elicit a protective immune response. This spontaneous immune reaction against 38C13 appears to be enhanced in the transductants. The xenoantigen(s) role as an adjuvant is supported by the finding of significantly higher titers of anti-Id antibodies in mice bearing s.c. 38C13-HER2/*neu* as compared to mice bearing s.c. 38C13 tumors.

We speculate that regression of 38C13-HER2/*neu* tumor was observed more frequently with larger tumor cell challenges because increased antigenic stimulation results from larger initial antigenic doses. This strong response may be able to eliminate the tumor before dissemination into the lymph nodes. In contrast, lower doses of cells fail to elicit efficient early stimulation of the immune response giving the tumors the

opportunity to disseminate and colonize most of the lymph nodes leading to their dysfunction. The secondary lymphoid organs are critical for both B and T cell mediated immunity (43) and their dysfunction would decrease the anti-tumor reaction allowing the tumor to progress. However, it should also be noted that effective anti-tumor responses are also not elicited by tumors that do not colonize the lymph nodes. It has been suggested that growing tumors are able to suppress the immune response against them (44). It is possible that in the absence of initial strong stimulation the growing tumor is able to decrease the subsequent immune response to its presence.

Our results suggest that under certain conditions expressing antigens of different species (xenoantigens) on B-cell lymphomas may be a useful therapeutic approach to eliciting an effective anti-tumor immune response. This can be achieved by vaccination with tumor cells expressing the xenoantigen, an approach that has shown benefits in ovarian cancer patients (45), or by *in vivo* transduction of B cell lymphomas with genes encoding xenoantigens. The presence of the foreign antigen on the surface of the B-cell lymphomas may induce strong stimulation of the patient's formerly quiescent immune system. Once stimulated, the patient's immune repertoire may be directed against other TAA resulting in the immune-mediated clearance of all B cell lymphoma cells.

The results obtained with s.c. implantation of cells contrast markedly with those obtained after i.v. injection of 38C13-HER2/*neu*. Following i.v. injection, all mice developed a disseminated malignant disease leading to death. Although the tumors elicited following i.v. injection maintain HER2/*neu* expression, they failed to elicit the strong anti-human HER2/*neu* and anti-Id humoral immune response observed in mice bearing s.c. 38C13 HER2/*neu*. This observation may not be surprising because it is well known that the s.c. route is superior to the i.v. route in eliciting an immune response in mice (46). Additionally, the i.v. injection of cells results in metastases to the lymph nodes throughout the body, which may seriously compromise the function of these

important secondary immune organs resulting in poor anti-tumor immunity (43) as discussed above.

38C13-HER2/*neu* may indeed be a useful model for evaluating the immunological efficacy of antibody or antibodies fusion proteins. We have shown that an effective immune response eliminates the tumor. This effective response is usually not elicited in mice injected s.c. with low doses (i.e. 10^3) of tumor cells or in mice injected i.v. with any dose. Tumors elicited following both s.c. and i.v. injection maintain high level of expression of human HER2/*neu.* In addition, the presence of anti-HER2/*neu* antibodies does not preclude the use of these mice for therapies targeting HER2/*neu* since the treatment can be started shortly after the injection of tumor cells when there is little or no humoral response. Indeed it may be possible to target the tumor in the presence of an anti-HER2/*neu* response since anti-human CEA was able to target MC38 transduced with human CEA (MC38-cea2) which elicits a murine anti-human CEA response (47). We also found that both anti-HER2/*neu* scFv and anti-HER2/*neu* IgG3 target CT26-HER2/*neu* growing in immunocompentent mice, a cell line also elicits a murine anti-human HER2/*neu* immune response (Penichet *et al*, unpublished results)[4]. We thus have the opportunity to use both HER2/*neu* and the Id as targets for our antibody fusion proteins. The challenge now is to develop a strategy that will make it possible to elicit an effective immune response to tumor after i.v. injection and/or to low doses of tumor cells injected s.c.

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Table 1 Survival of C3H/HeN mice challenged with different doses of 38C13 or 38C13-HER2/*neu*.

Tumor injection	Number of mice	Number of survivors
Experiment 1		
10^3 38C13-HER2/*neu*	5	1
10^4 38C13-HER2/*neu*	5	4
10^5 38C13-HER2/*neu*	5	4
10^6 38C13-HER2/*neu*	5	5
10^3 38C13	5	0
10^4 38C13	5	0
10^5 38C13	5	0
10^6 38C13	5	1
Experiment 2		
10^3 38C13-HER2/*neu*	10	2
10^4 38C13-HER2/*neu*	10	4
10^5 38C13-HER2/*neu*	10	4
10^6 38C13-HER2/*neu*	20	16
10^3 38C13	10	0
10^4 38C13	10	0
10^5 38C13	10	0
10^6 38C13	20	2

Table 2 Survival of mice challenged with 38C13 or V1 cells.

C3H/HeN mice that showed complete tumor rejection following injection with 38C13-HER2/*neu* were challenged with a lethal dose (10^4) of 38C13 or V1, an Id-negative variant derived from 38C13. The challenge was made two months after the primary tumor had completely regressed. As controls, the same number of naïve C3H/HeN mice of similar age were injected with 38C13 or V1.

Previous dose of injection	Challenge	Number of mice	Number of survivors
10^3	38C13 (10^4)	3	2
10^4	38C13 (10^4)	5	5
10^5	38C13 (10^4)	7	7
10^6	38C13 (10^4)	20	20
None (controls)	38C13 (10^4)	35	0
10^6	V1 (10^4)	8	8
None (controls)	V1 (10^4)	8	0

Table 3 Kinetics of anti-human HER2/*neu* and anti-Id Ab response in mice bearing 38C13-HER2/*neu* or 38C13 tumors.

Groups of 5 mice were injected in the right flank with 10^4 38C13-HER2/*neu* or 38C13 cells. Mice were bled every three days and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or Id. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm). Tumor growth was monitored and measurements were recorded three times per week with a caliper and was classified as progression (P) or regression (R).

Cells injected	Antibody tested	Mouse number	Days after challenge					Tumor response
			3	6	9	12	15	
38C13-HER2/*neu*	anti-human HER2/*neu*	1	0	50	150	50	4050	R
		2	0	0	50	450	1350	R
		3	0	0	0	1350	1350	R
		4	0	0	50	50	450	P
		5	0	50	150	1350	4050	R
	anti-mouse Id	1	0	0	0	50	1350	R
		2	0	0	0	450	1350	R
		3	0	0	0	150	450	R
		4	0	0	0	0	0	P
		5	0	0	0	0	0	R
38C13	anti-mouse Id	1	0	0	0	0	0	P
		2	0	0	0	0	0	P
		3	0	0	0	0	0	P
		4	0	0	0	0	0	P
		5	0	0	0	0	0	P

Table 4 Comparison of anti-human HER2/*neu* and anti-Id levels in mice bearing s.c. 38C13-HER2/*neu* or 38C13 tumors.

Groups of 5 mice injected in the right flank with 10^3, 10^4,10^5 or 10^6 of 38C13-HER2/*neu* or 38C13 cells were bled 12 days after the injection of the cells and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or Id. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm). Tumor growth was monitored and measurements were recorded three times per week with a caliper. Growth was classified as progression (P) or regression (R).

Cells injected	Antibody tested	Mouse number	Dose of cells & tumor response			
			10^3	10^4	10^5	10^6
38C13-HER2/*neu*	anti-human HER2/*neu*	1	0 P	450 P	4050 P	450 R
		2	0 P	150 R	450 R	450 P
		3	0 R	50 P	1350 P	1350 R
		4	0 R	50 R	450 P	450 R
		5	0 P	450 R	450 P	450 R
	anti-mouse Id	1	0 P	50 P	150 P	450 R
		2	0 P	150 R	450 R	150 P
		3	0 R	0 P	50 P	450 R
		4	0 R	50 R	150 P	1350 R
		5	0 P	0 R	0 P	450 R
38C13	anti-mouse Id	1	0 P	0 P	150 P	50 P
		2	0 P	0 P	150 P	450 P
		3	0 P	0 P	0 P	150 P
		4	0 P	0 P	0 P	150 P
		5	0 P	0 P	50 P	150 P

Table 5 Comparison of anti-human HER2/*neu* and anti-Id levels in mice injected i.v. with 38C13-HER2/*neu* cells.

Groups of 5 mice injected by the vein tail with 10^3, 10^4,10^5 or 10^6 of 38C13-HER2/*neu* cells were bled 12 days after the injection of the cells and the sera analyzed by a titration ELISA using plates coated with the ECD^{HER2} or Id. The presence of Abs was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm).

Cells injected	Antibody tested	Mouse number	Dose of cells			
			10^3	10^4	10^5	10^6
38C13-HER2/*neu*	anti-human HER2/*neu*	1	0	150	50	0
		2	50	Dead	Dead	150
		3	150	150	50	50
		4	50	Dead	150	50
		5	450	50	50	150
	anti-mouse Id	1	0	0	0	50
		2	0	Dead	Dead	0
		3	0	0	0	0
		4	0	Dead	0	150
		5	0	0	0	50

Figure Legends

Fig. 1. Analysis by flow cytometry of the surface expression of human HER2/*neu* and Id by 38C13-HER2/*neu* (panels A, B and C) or 38C13 (panels D, E and F). Cells were stained with anti-DNS human IgG3 (panels A and D), anti-HER2/*neu* human IgG3 (panels B and E) or anti-Id human IgG3 (panels C and F), followed by biotinylated goat anti-human IgG and PE-labeled streptavidin.

Fig. 2. Kinetics of tumor growth. Groups of 5 mice were injected in the right flank with 10^3,10^4, 10^5 or 10^6 cells from either 38C13 or 38C13-HER2/*neu*. Tumor growth was monitored and measurements were recorded three times per week with a caliper.

Fig. 3. Survival of C3H/HeN mice (5/group) inoculated s.c. with 10^3 (○), 10^4 (●), 10^5 (□), or 10^6 (■) 38C13 (panel A) or 38C13-HER2/*neu* (panel B).

Fig. 4. Histologic sections of a 12 day old continously growing 38C13 tumor (panel A) or a 12 day old regressing 38C13-HER2/*neu* tumor (panel B). Both tumors were from C3H/HeN mice injected s.c. with 10^6 tumor cells. The histologic study was carried out on paraformaldehyde-fixed, paraffin-embedded 6 μm sections stained with hematoxylin/eosin. Bar = 100 μm.

Fig. 5. Survival of C3H/HeN mice challenged s.c. with a lethal dose of 10^4 38C13. One day before challenge, groups of 8 mice each received a single i.v. injection of 5 x 10^7 spleenocytes from immune animals previously inocualted with 38C13-HER2/*neu* (Δ), 0.3 ml of serum from immune animals (□) or untreated animals (○).

Fig. 6. Survival of C3H/HeN mice (5/group) inoculated i.v. with 10^3 (○), 10^4 (●), 10^5 (□), or 10^6 (■) 38C13 (panel A) or 38C13-HER2/*neu* (panel B).

Fig. 7. Flow cytometry analysis of 38C13-HER2/*neu* cells freshly isolated from tumors of two mice injected s.c. with 10^4 cells (panels A and B) or from cervical metastasis of two mice injected i.v. with 10^4 cells (panels C and D). All tumors were harvested 15 days after tumor cell injection. The cells were stained with anti-dansyl human IgG3 (dotted line) or anti-HER2/*neu* human IgG3 (thick solid line), followed by biotinylated goat anti-human IgG and PE-labeled streptavidin. The expression of human HER2/*neu* was compared with the expression detected in 38C13-HER2/*neu* cells maintained in culture (thin solid line). We also tested the expression of HER2/*neu* after the cells were cultured *in vitro* for 1 week. Panels E-H represent the cells shown in panels A-D respectively after they were maintained for one week in tissue culture.

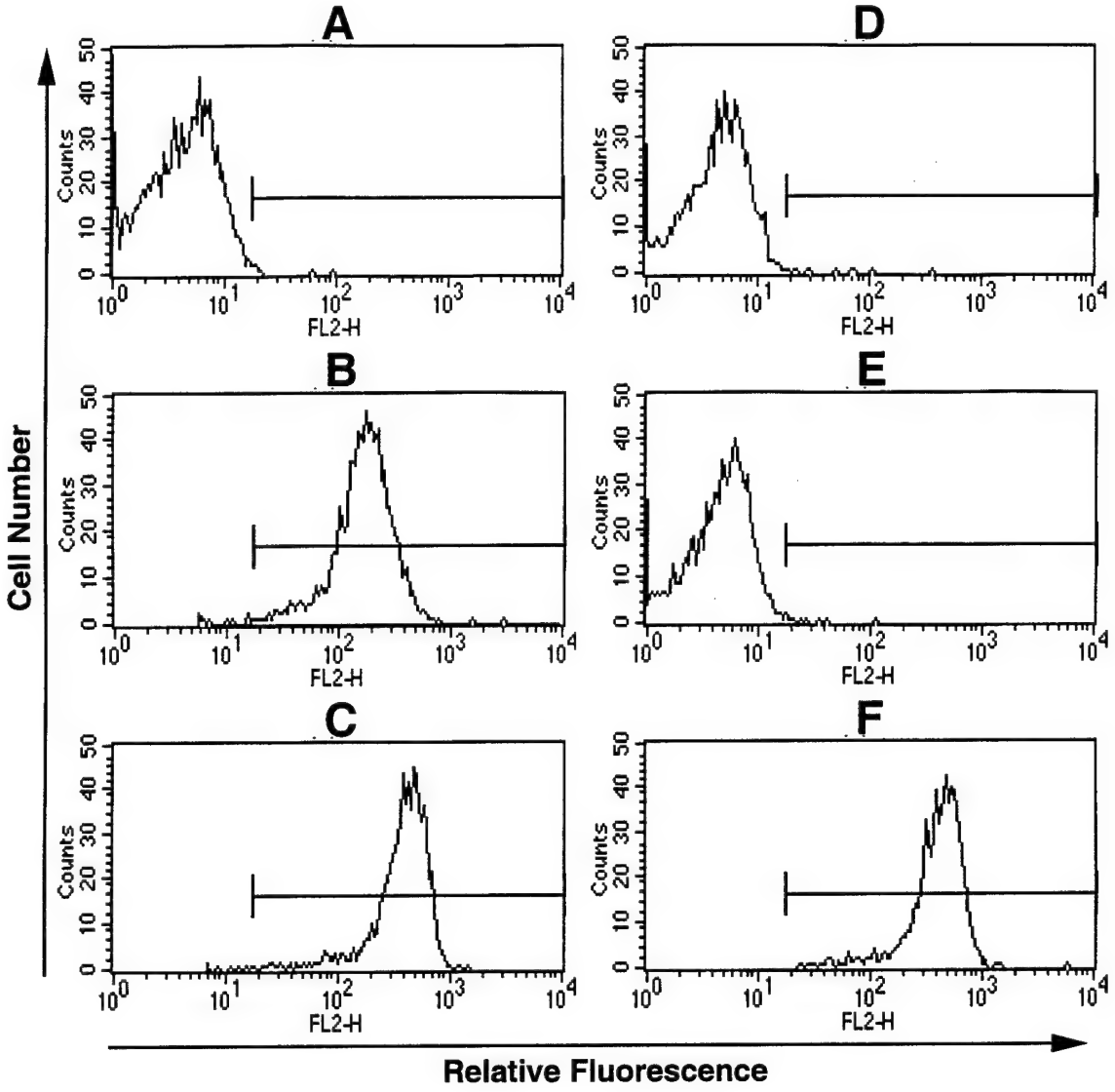

Figure 1

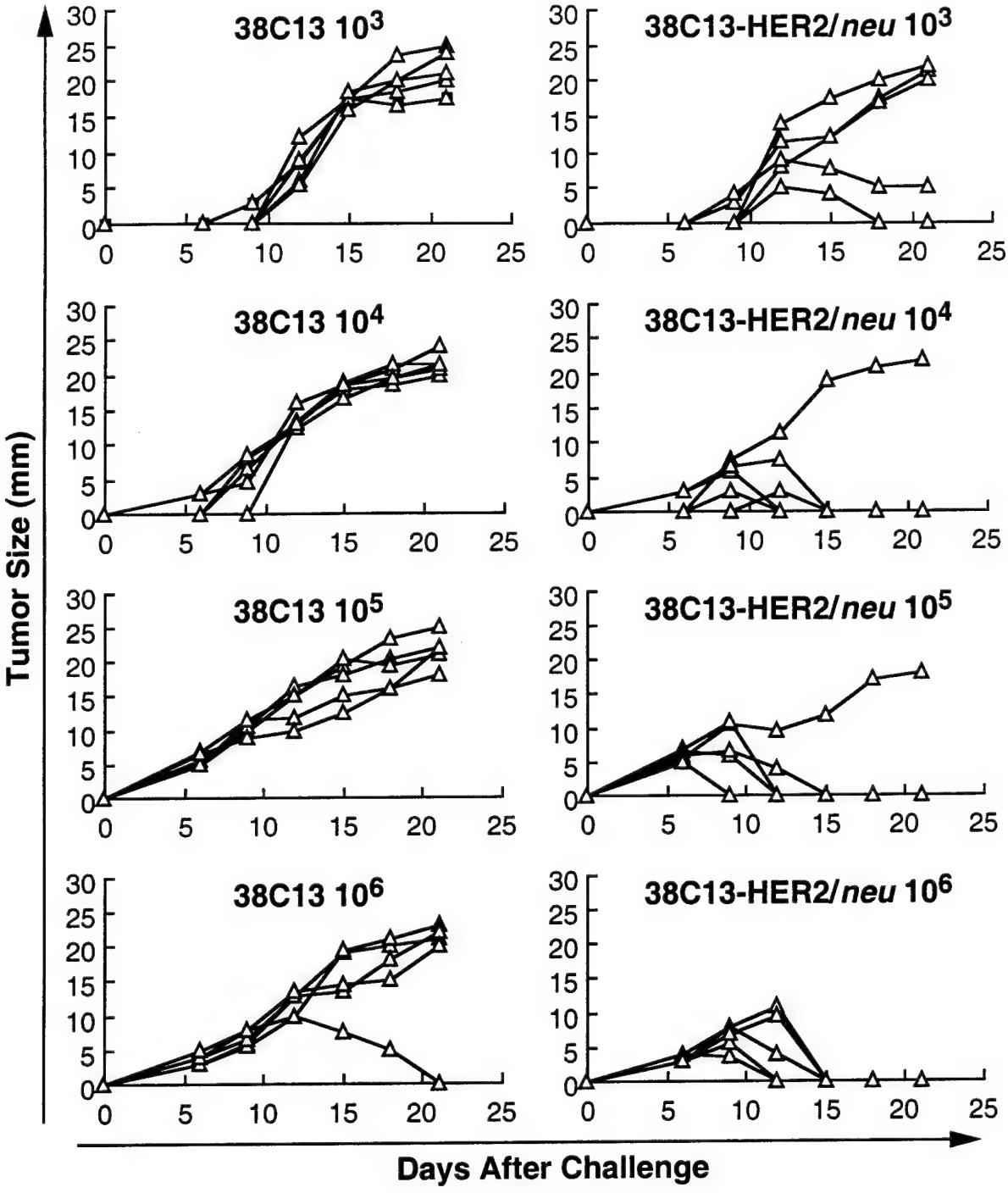
38C13 10³
38C13-HER2/neu 10³
38C13 10⁴
38C13-HER2/neu 10⁴
38C13 10⁵
38C13-HER2/neu 10⁵
38C13 10⁶
38C13-HER2/neu 10⁶
Tumor Size (mm)
Days After Challenge
0
5
10
15
20
25
30

Figure 2

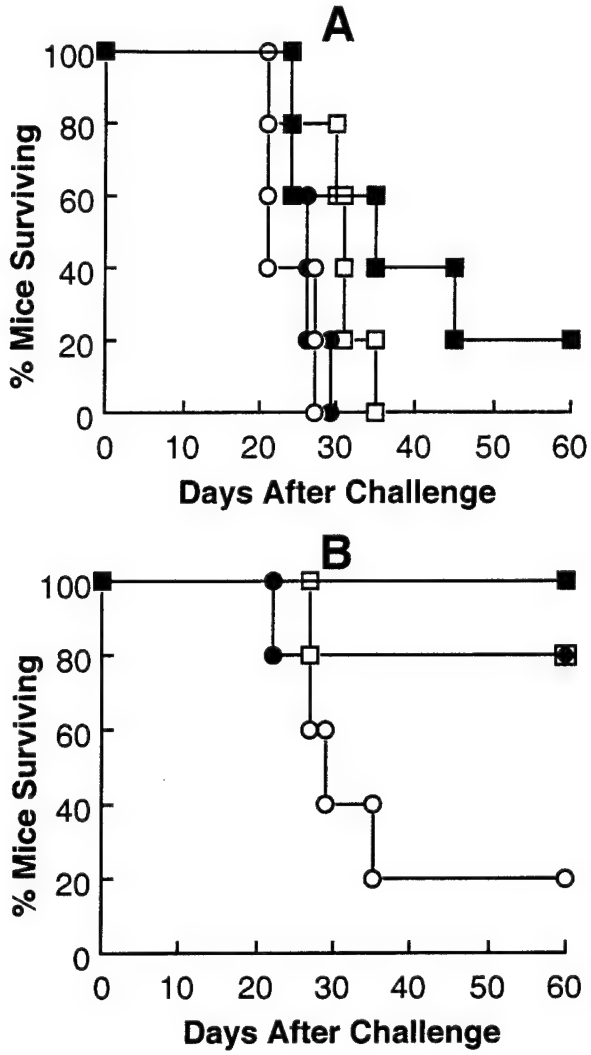
A
100
80
60
40
20
0
% Mice Surviving
0
10
20
30
40
50
60
Days After Challenge
B
100
80
60
40
20
0
% Mice Surviving
0
10
20
30
40
50
60
Days After Challenge

Figure 3

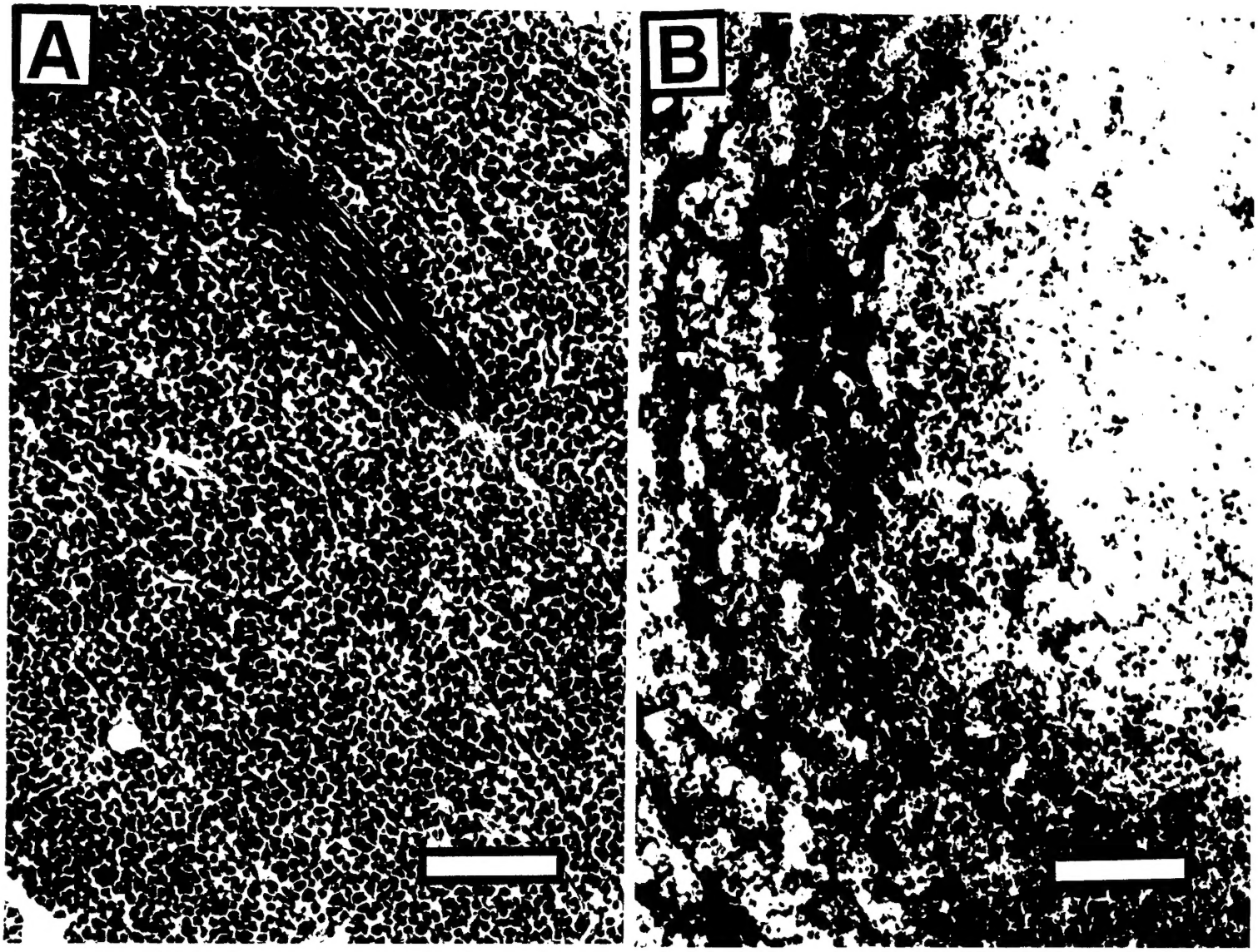

Figure 4

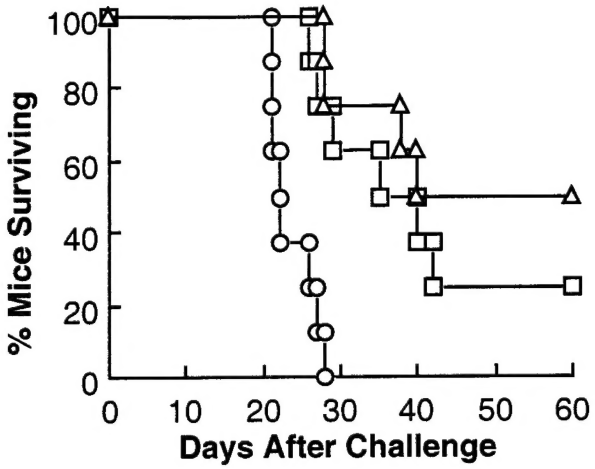
% Mice Surviving
100
80
60
40
20
0
0
10
20
30
40
50
60
Days After Challenge

Figure 5

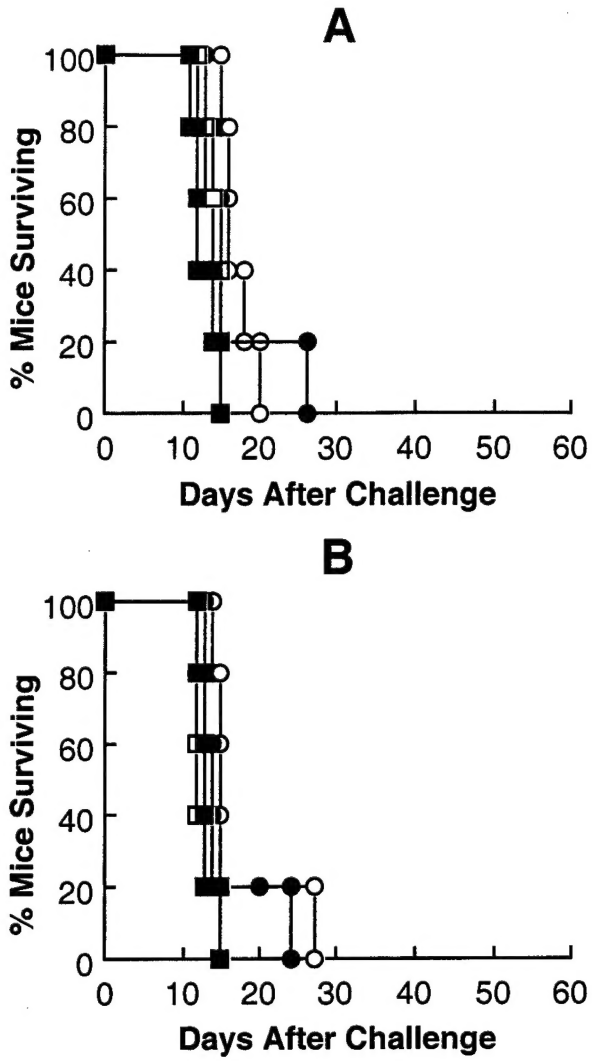
A
100
80
60
40
20
0
% Mice Surviving
0
10
20
30
40
50
60
Days After Challenge
B
100
80
60
40
20
0
% Mice Surviving
0
10
20
30
40
50
60
Days After Challenge

Figure 6

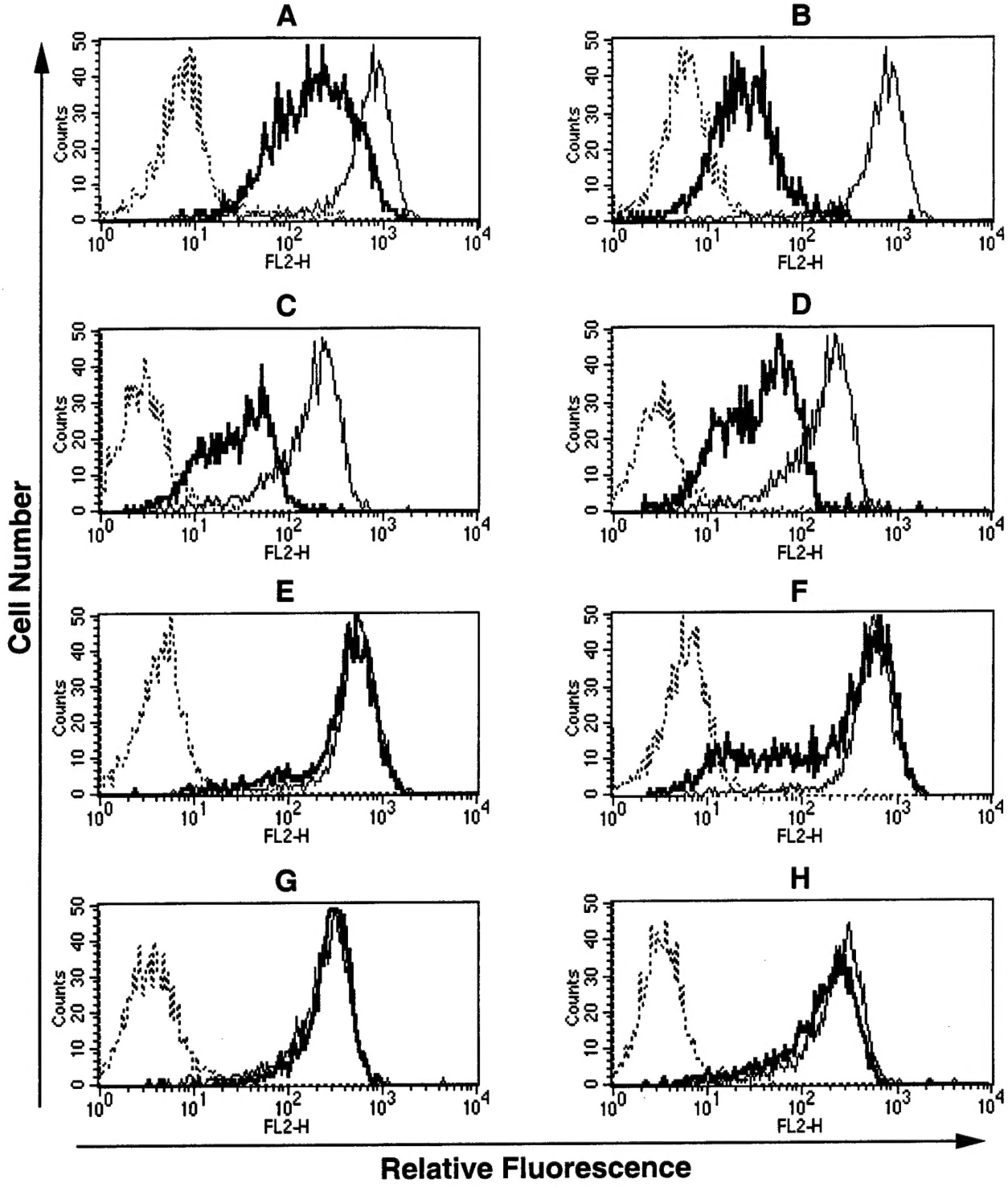

Figure 7